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PHYSIOLOGICAL AND TUMORAL CONSEQUENCES OF AURORA B DEREGULATION IN ADULT MAMMALS

TESIS DOCTORAL

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Physiological and tumoral consequences of Aurora B deregulation in adult mammals

Memoria presentada por Alejandra González Loyola, licenciada en Biología y Bioquímica, bajo la dirección del doctor Marcos Malumbres, para optar al grado de Doctor Internacional.

Centro Nacional de Investigaciones Oncológicas (CNIO)

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Marcos Malumbres Martínez, Jefe del Grupo de División Celular y Cáncer del Centro Nacional de Investigaciones Oncológicas (CNIO)

CERTIFICA

Que la tesis doctoral titulada "Physiological and tumoral consequences of Aurora B deregulation in adults mammals" ha sido realizada en el Centro Nacional de Investigaciones Oncológicas y tutelada en el Departamento de Biología Molecular de la Universidad Autónoma de Madrid.

El trabajo realizado por Alejandra González Loyola reúne todas las condiciones requeridas por la legislación vigente, así como la originalidad y calidad científica para poder ser presentada y defendida con el fin de optar al grado de Doctor Internacional por la Universidad Autónoma de Madrid.

Y para que así conste, firmo el presente certificado

Madrid, a 18 de diciembre de 2014

Dr. Marcos Malumbres Martínez
Tutor y Director de la Tesis

A mis padres y mis abuelos,
a Laurita, Nastia y Cris
y a mis tíos

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Madrid, a 18 de diciembre de 2014

Good things come to those who wait, a maxim that is as true for cells as it is elsewhere in life.

A

APC/C:	Anaphase- Promoting Complex/ Cyclosome
Aurka:	Aurora kinase A (gene)
Aurkb:	Aurora kinase B (gene)
Aurkc:	Aurora kinase C (gene)

B

BAC:	Bacterial Artificial Chromosome
BAT:	Brown Adipose Tissue
BrdU:	Bromodeoxiuridine
BM:	Bone Marrow

C

Cdc20:	Cell Division Cycle 20
cDNA:	Complementary DNA
Cdk:	Cycle-dependent kinase
CIN:	Chromosomal Instability
CMV:	Cytomegalovirus promoter
CPC:	Chromosomal Passenger Complex

D

DAPI:	4',6-diamidino-2-phenylindole
DMEM:	Dulbecco's modified Eagle's medium
DMSO:	Dymethyl sulfoxide
DEXA:	Dual Energy X-Ray Absortimetry
Doxy:	Doxycycline

E

EE:	Energy expenditure
ES cells:	Embryonic Stem cells
EV:	Empty Vector

F

FACS:	Fluorescence Activated Cell Sorting
FBS:	Fetal Bovine Serum

G

G0:	Gap phase 0
G1:	Gap phase 1
G2:	Gap phase 2
GADPH:	Glyceraldehide 3-phosphate dehydrogenase
GFP:	Green Fluorescent Protein
GTPase:	Guanine nucleotide triphosphatase
γ-H2AX:	Gamma histone H2AX

H

H&E:	Hematosilin and Eosin
HR:	Homologous Recombination

I

IF:	Inmunofluorescence
IHC:	Inmunohistochemistry
iPCs:	Induced pluripotent cells

K

KD:	Kinase-Dead

M

MCC:	Mitotic Checkpoint Complex
MEFs:	Mouse Embryonic Fibroblasts

N

Noc:	Nocodazole

P

PBS:	Phosphate-Buffered Saline
pH3:	phospho-Histone H3
Plk1:	Polo-like kinase 1

Q

qRT-PCR:	quantitative Reverse Transcription Polymerase Chain Reaction

R

rTTA:	reverse Tetracycline Transactivator
RQ:	Respiratory quotient

S

SAC:	Spindle Assembly Checkpoint

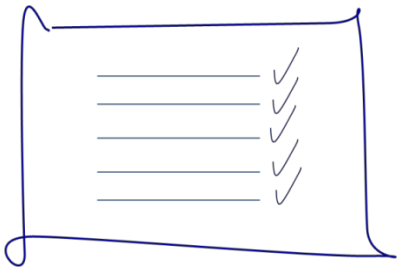
T

Tet:	Tetracycline
TetO:	Tetracycline Operator Elements
Tet-P:	Tetracycline-responsive minimal promoter
Tg:	Transgenic mice

W

WAT:	White Adipose Tissue
WB:	Western Blot
WT:	Wild Type

Index



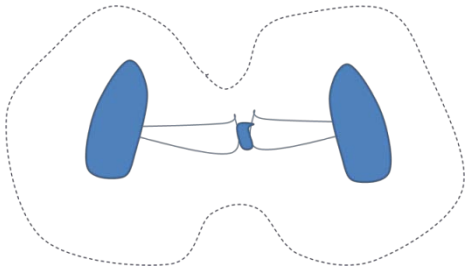
Summary/Resumen

Summary

Aurora B (*Aurkb*), one of the three members of the mammalian Aurora kinase family, is the catalytic component of the Chromosomal Passenger Complex (CPC), an essential regulator of chromosome segregation in mitosis. Despite the numerous studies on Aurora B function in cells, the physiological effects of Aurora B deregulation *in vivo* are unknown. In this work we have analysed conditional genetic depletion and overexpression mouse models for *Aurkb* that allowed us to modulate Aurora B expression in murine tissues and cells. Aurora B is indispensable for cell proliferation except during the initial stages of embryonic development in which it is substituted by Aurora C (*Aurkc*). Its widespread elimination in adult tissues led to an ageing phenotype characterized by proliferative defects and aneuploidy. The observed mitotic arrest in tissues was a consequence of less regenerative capacity of adult stem cells. This was accompanied by a stress response characterized by the induction of p53 and $p21^{Cip1}$. On the other hand, *Aurkb* is overexpressed in human tumours although whether this kinase may function as an oncogene *in vivo* is not established. Here, we take advantage of a new mouse model in which the expression of the endogenous *Aurkb* locus can be induced *in vitro* and *in vivo*. Overexpression of Aurora B in cultured cells induced defective chromosome segregation and aneuploidy. Long-term overexpression of Aurora B *in vivo* resulted in aneuploidy and the development of multiple spontaneous tumours in adult mice including high incidence of lymphomas. In line with previous reports linking Aurora B with p53 activity, overexpression of Aurora B resulted in reduced DNA damage response and decreased levels of the p53 target $p21^{Cip1}$ *in vitro* and *in vivo*, in concordance with an inverse correlation between Aurora B and $p21^{Cip1}$ expression in human leukaemias. Thus, overexpression of Aurora B may contribute to tumour formation not only by inducing chromosomal instability but also suppressing the function of the cell cycle inhibitor $p21^{Cip1}$. In addition, overexpression of Aurora B *in vivo* resulted in the deposit of white adipose tissue (WAT) inside the Brown adipose tissue (BAT), liver steatosis and in adipose hyperplasia, which suggests a role for Aurora B outside mitosis. Finally, since Aurora C is the kinase that drives mitosis in early zygotes, we have analysed its role in stemness potential. Our data suggest that Aurora C is induced during the reprogramming process and that its upregulation results in enhanced number of stem cells colonies during reprogramming accompanied by deregulation of specific epigenetic marks. These data suggest specific functions for Aurora C in the pluripotent condition.

Resumen

La quinasa Aurora B (*Aurkb*), uno de los tres miembros de la familia de quinasas Aurora, es el componente catalítico del complejo pasajero de los cromosomas (CPC), un regulador esencial de la segregación cromosómica durante el proceso de mitosis. Aunque su función celular se ha estudiado en detalle, los efectos fisiológicos de su desregulación *in vivo* se desconocen. En este trabajo hemos analizado dos modelos animales para Aurora B, uno de elimación y otro de sobre-expresión que nos han permitido modular la expresión de Aurora B en células y tejidos de ratón. Aurora B es indispensable para una adecuada proliferación celular excepto durante las primeros estadíos del desarrollo embrionario en los que es sustituida por Aurora C. Su eliminación en tejidos adultos lleva a un fenotipo de envejecimiento prematuro, caracterizado por defectos en proliferación y aneuploidía. El arresto mitótico observado en los tejidos es consecuencia de una menor capacidad regenerativa de las células madre adultas. Además observamos una respuesta al estrés caracterizada por una inducción de p53 y $p21^{Cip1}$. Por otra parte, Aurora B está sobre-expresada en tumores humanos aunque no está establecido si puede funcionar como un oncogen. Gracias a la generación de un nuevo modelo animal inducimos la expresión del gen endógeno de Aurora B *in vitro* e *in vivo*. La sobre-expresión de Aurora B en células induce defectos en la segregación cromosómica y aneuploidía y a largo plazo en ratones adultos provoca aneuploidia y múltiples tumores, predominantetmente linfomas. En consonancia con resultados anteriores que correlacionan Aurora B con la actividad de p53, la sobre-expresión de Aurora B provoca una menor respuesta al daño al DNA y una disminución en los niveles de la diana de p53, el inhibidor del ciclo celular $p21^{Cip1}$, *in vitro* e *in vivo*. Datos que están en consonancia con la correlación inversa en cuanto a la expresión de Aurora B y $p21^{Cip1}$ en leucemias humanas. Además los ratones que sobre-expresan Aurora B presentan tejido adiposo blanco en el tejido adiposo marrón, esteatosis en hígado e hiperplasia de adipocitos, lo que indica un posible papel de Aurora B fuera de mitosis. Finalmente, debido a que Aurora C es la quinasa encargada de dirigir la mitosis durante los primeros estadíos embrionarios decidimos analizar el papel de Aurora C en la generación de la condición pluripotente. Observamos su activación durante la reprogramación celular además de un aumento en la eficiencia de formación de colonias y desregulación de marcas epigenéticas cuando sobre-expresamos Aurora C, sugiriendo funciones específicas de esta quinasa en el mantenimiento del potencial pluripotente.



Introduction

Introduction

1. The mammalian Cell Division Cycle

"*Omnis cellula e cellula*", an important dogma in cell biology, was born when Rudolf Virchow in 1858 established that every cell must derive from a pre-existing cell. And indeed cell division is the only way for life to expand and, unfortunately, when uncontrolled also the way for cancer. Cell division cycle describes a series of events that ensures faithfully the transition of the genetic information from one cell generation to the next. The majority of mammalian adult cells are in a quiescent state called G0, and only when they are exposed to specific mitogenic stimuli or signaling cells enter the cell cycle. In eukaryotic cells, the cell cycle was first described as two distinct phases: interphase and mitosis. Interphase was later on divided into three phases, S-phase standing for synthesis of DNA, surrounded by two Gap-phases G1 (gap1) and G2 (gap2). The stage for the synthesis of DNA in which the cell prepares and grows is G1, next during S-phase the cell replicates its genetic material and in G2 the cell prepares for its division. During mitosis (M phase) the cell segregates its DNA into two daughter cells (Figure 1).

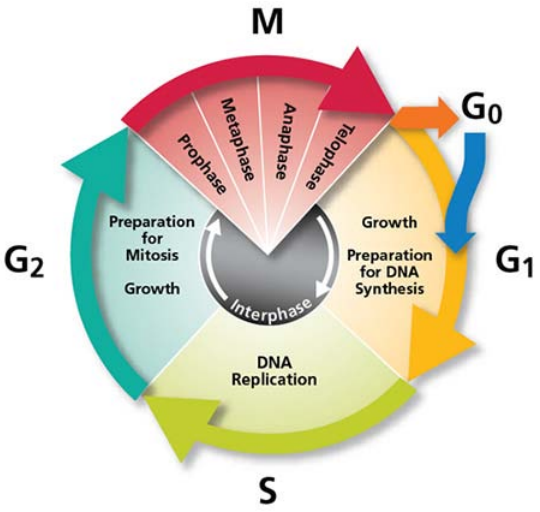


Figure 1. Cell cycle phases. Quiescent cells in G0 decide to cycle and enter into G1 to prepare for the DNA synthesis in S phase. Once the genome is duplicated cells prepare to divide in G2. Finally, chromosome segregation takes place in M phase and cell divides (Adapted from BD Biosciences resources).

Mitosis, described by Walter Flemming in 1882, is the nuclear division process in which the previously duplicated genome is reorganized into compact chromosomes, each made up of two sister chromatids that are equally segregated into two daughter cells. It is the most spectacular and sophisticated part of the cell cycle. In less than an hour the mother cell organizes a complex machinery aiming to have each daughter cell inherit a complete set of chromosomes, a centrosome (the main microtubule-organizing centre of animal cells) the cytoplasm and organelles. Mitosis is characterized by five different phases: prophase (P), prometaphase (PM), metaphase (M), anaphase (A) and telophase (T) (Figure 2).

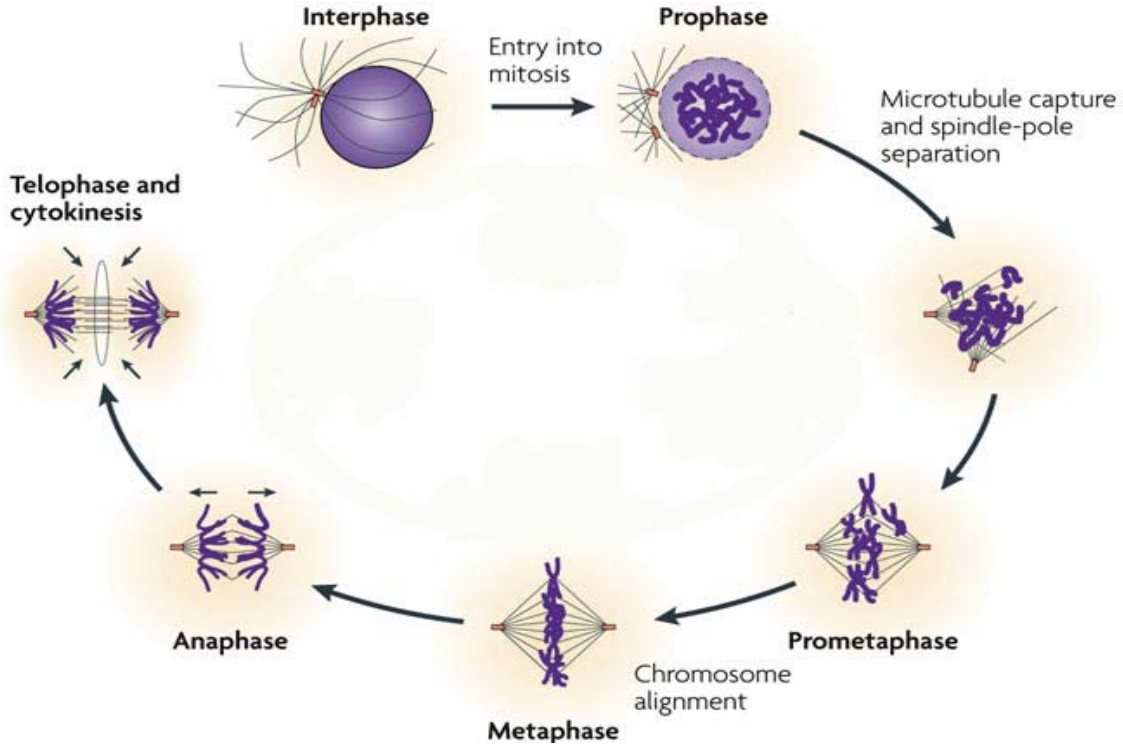


Figure 2. The phases of Mitosis. Interphasic chromatin is condensed and kinetochores assemble in prophase. In prometaphase, nuclear envelope breakdown and kinetochores bind to microtubules. Chromosomes are bioriented and aligned during metaphase in the spindle midzone, forming a metaphase plate. In anaphase, sister chromatids are pulled apart. At telophase, chromatin decondenses and the nuclear envelope is reformed. During cytokinesis, the cortex constricts under the action of the actomyosin ring (Adapted from (Salaun et al., 2008).

During prophase, interphasic chromatin starts to condense into chromosomes that move to the poles of the cell where the spindle structure will be formed. Then, prometaphase begins with the nuclear envelope breakdown and the mitotic spindle formation; a dynamic bipolar array of microtubules. The spindle microtubules are then captured by the chromosome's kinetochores, a proteinaceous structure located on the centromeres of chromosomes. Microtubules from opposite poles interact with chromosomes and make them become bioriented and congressed, reaching the equator of the spindle thus forming the "metaphase plate". Once all the chromosomes are properly bioriented, a loss of sister-chromatin cohesion triggers the anaphase step. Early in anaphase, chromosomes lose their cohesion and each chromatid moves apart towards one spindle pole. At late anaphase, the spindle is elongated and separates further the two set of chromatids. In telophase, the two daughter chromosomes reach the spindle poles, chromatin decondenses and the nuclear envelope is reformed around the two daughter chromosomes. Then, the cytoplasm division, also called cytokinesis and whose regulation is precisely linked to mitosis, occurs. A contractile ring is formed at the cortex of the cell giving rise to the midbody that marks the abscission site. This is the final step of cell division that physically separates the two daughter cells from each other (Glotzer, 2005; Guertin et al., 2002) (Figure 2).

2. Cell Cycle Checkpoints

Dividing cells must wait until their DNA is completely duplicated before they can segregate the replicas. They must also delay the next round of DNA replication until the copies are safely deposited in two newly born daughter cells. The order and completion of these events are crucial; otherwise, chromosomes become unstable, and processes such as cancer may result. To ensure their proper progression through the cell cycle, cells have developed a series of checkpoints that prevent them from entering into a new phase until they have successfully completed the previous one (Malumbres and Barbacid, 2001).

A quiescent cell has first to decide whether to cycle or not. The cell has to balance the mitogenic and anti-mitogenic signals to decide whether to enter or not into the cell cycle (Pardee, 1974). Once cells reach the restriction point they are committed to enter mitosis and replicate its genome. This decision is crucial since cycling under improper conditions may cooperate with tumour progression. Indeed, cancer cells are characterized by alterations in their signal transduction pathways leading to overproliferation (Hanahan and Weinberg, 2000).

Moreover, the genome must remain stable to prevent uncorrected processes such as malignant cell transformation. Mammalian cells display DNA damage checkpoints in order to sense any kind of damage to the DNA and transduce the signal to activate the p53 and retinoblastoma (pRb) tumour suppressor pathways. These pathways allow the cell with time to repair its DNA avoiding its entrance into mitosis until the damage has been repaired (Bartek et al., 2004); (Kastan and Bartek, 2004) (Lukas et al., 2006). The DNA damage checkpoint can arrest cell cycle progression during G1/S, S phase or at G2/M stage.

2.1. The Spindle Assembly Checkpoint

Cells have developed a surveillance mechanism to monitor kinetochore-microtubule attachment called the spindle assembly checkpoint (SAC), which delays the onset of anaphase until all chromosomes are properly bioriented. If a single kinetochore is not attached to the spindle, the spindle checkpoint is activated, thus maintaining the fidelity of chromosome segregation (Musacchio and Hardwick, 2002). (Figure 3).

The SAC is an important safeguard mechanism that ensures faithful chromosome segregation in eukaryotic cells. The effector of the SAC is known as the mitotic checkpoint complex (MCC), it is located at unattached kinetochores and is composed by the mitotic arrest deficient 2 protein (Mad2), mitotic checkpoint serine/threonine-protein kinase BUB1 beta (BubR1) and the mitotic checkpoint protein Bub3 (Sudakin et al., 2001)

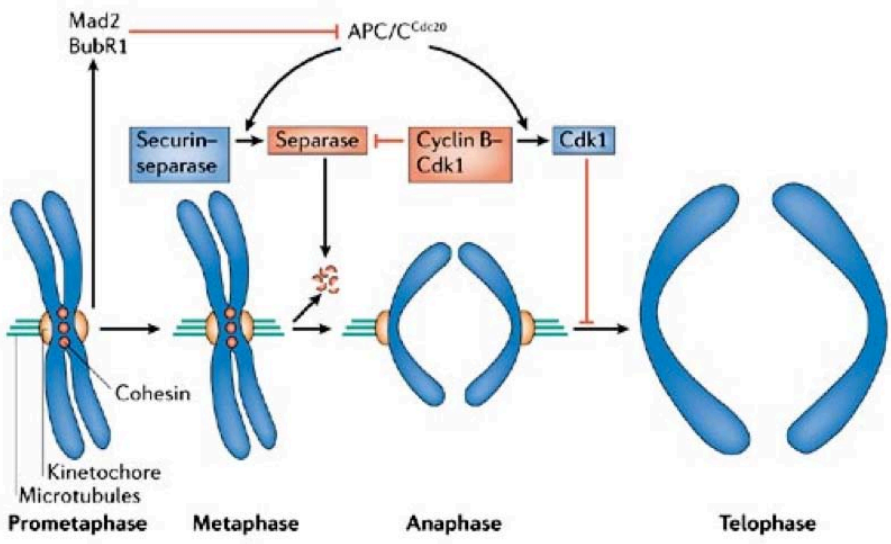


Figure 3. The Spindle Assembly Checkpoint. During PM to A, kinetochores catalyze the formation of the MCC, composed of Mad2, BubR1, Bub3 and Cdc20, leading to the inhibition of the APC/C. Once all the chromosomes are aligned at the metaphase plate, MCC allows Cdc20 to activate the APC/C leading to the degradation of cyclin B1 and securin. Securin degradation liberates separase which cleaves the cohesion ring structure allowing sister chromatids to separate , while cyclin B1 degradation allows Cdk1 inactivation and exit from mitosis (Peters, 2006).

The MCC inhibits the cell division cycle 20 (Cdc20), a protein that activates the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C). Once the chromosomes are properly bioriented, the SAC is satisfied and Cdc20 is free to activate the APC/C-polyubiquitinilation of two substrates, cyclin B1 and securin. Securin is an inhibitor of a protease known as separase, which cleaves the cohesion complex that holds sister chromatids together, a process that is necessary to execute anaphase. Cyclin B1 proteolysis is required to inactivate Cdk1 and thus exit mitosis (Figure 3).

3. Control of mitosis and cytokinesis by phosphorylation

Cell cycle progression requires the controlled activation of different families of serine/threonine kinases that by phosphorylation regulate different cellular processes to ensure a proper segregation of chromosomes. The cyclin-dependent kinases (Cdks) are heterodimeric proteins composed of a catalytic subunit (Cdk) and a regulatory subunit, known as cyclin. There are twenty Cdks that must associate to a cyclin to become active kinases. Cyclins expression and degradation impose waves of kinase activation and inactivation that control cell cycle commitment, DNA synthesis and mitosis onset (Malumbres and Barbacid, 2005). To enter into mitosis, Cdk1 must be activated by A-type cyclins during prophase and by cyclin-B after the nuclear envelope breakdown (Draetta et al., 1989; Karaiskou et al., 2001). The complex formed by Cdk1/cyclin B which is known as the Maturation Promoting Factor (MPF) is consider as the

master regulator at the onset of mitosis due to its role in key processes such as nuclear envelope breakdown, centrosome separation and chromosome condensation between others. This complex must be inactivated to exit from mitosis upon SAC inactivation and Cyclin B degradation by the APC/C (Malumbres, 2011).

The DNA damage kinases have also a role in mitosis. The main DNA damage kinases are Atm (Ataxia-Telangiectasia Mutated) and Atr (Atm and Rad3 related) and the checkpoint kinases Chk1 and Chk2. When they are activated, they regulate kinases such as Wee1 and Myt1, which inactivate Cdks or phosphatases such as Cdc25 family, which activate Cdks by removing their previous phosphorylations (Malumbres, 2011).

Mitotic kinases are the kinases of the Polo, Nek and Aurora families that regulate centrosome cycle, spindle functioning and chromosome segregation during mitosis. Polo-like (Plk) family is characterized by the presence of a specific domain, known as the polo box (PBD), involved in protein-protein interactions and is formed by five members in mammals, Plk1 being the best characterized. Plk1 activates mitotic entry by phosphorylating Cdk-cyclin B. Plk1 is also implicated in centrosome maturation, cohesion release in prophase and cytokinesis initiation (Barr et al., 2004). Plk2 and Plk3 are mitogen activated kinases with tumour suppressor functions (Eckerdt et al., 2005; Kleylein-Sohn et al., 2007). Plk1 to Plk4 are involved in centrosome biology as well as the regulation of mitosis, cytokinesis, and cell cycle checkpoints in response to genotoxic stress, whereas Plk5 is a new member of the vertebrate family, that has evolved as a kinase-deficient PBD-containing protein with nervous system-specific functions and tumour suppressor activity in brain cancer (de Cárcer et al., 2011).

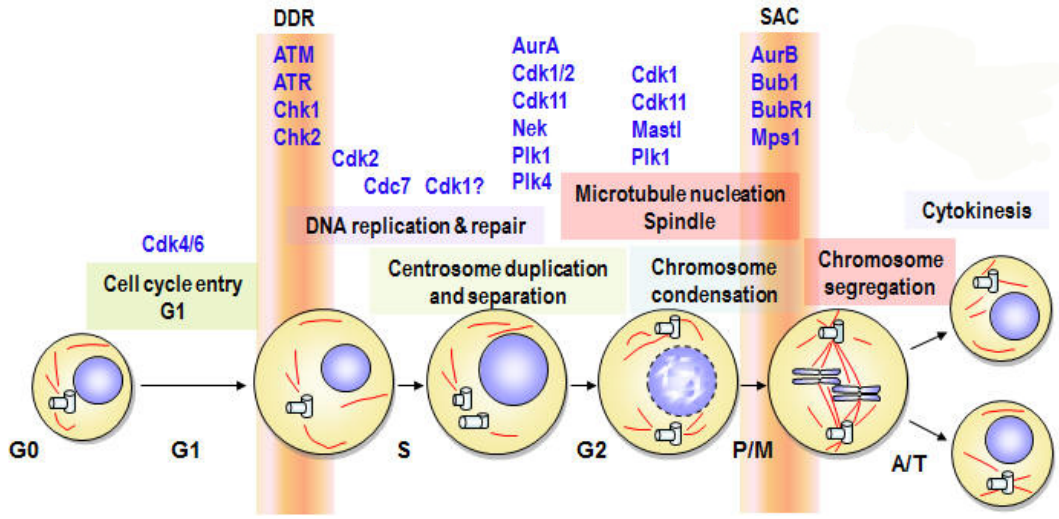


Figure 4. Overview of mammalian cell cycle kinases. A typical cell is represented that grows in size after entering the cell cycle. During the S phase the genome (blue) is duplicated and chromosomes condensate during mitosis to be equally segregated between the two daughter cells. Microtubules are represented as red lines and centrioles as small cylinders (Modified from (Malumbres, 2011).

The NIMA-related kinase (Nek) family consists on eleven members being Nek2 the best known (O'Connell et al., 2003). Nek2 is known to be a core component of the human centrosome and crucial for cytokinesis (Fry, 2002). Within the group of mitotic kinases, the Aurora kinase family includes key mitotic regulators that will be described in detail in the fifth section.

There are also crucial kinases implicated in SAC regulation such as BubR1, the Mitotic checkpoint serine/threonine-protein kinase Bub1 (budding uninhibited by benzimidazoles 1) and Monopolar spindle 1 (Mps1). Their function is also tightly linked to their localizations during mitotic progression, "being at the right place at the right time" (Figure 4). BubR1 associates and directly phosphorylates Cdc20, similar to Mad2 (Skoufias et al., 2001; Tang et al., 2001). BubR1 and Mad2 enhance each other's ability to bind to Cdc20 and they function synergistically in inhibiting APC, thus leading to a complete arrest of mitotic progression (Fang, 2002).

4. Chromosomal instability and Cancer

4.1. Chromosomal Instability

Chromosome Instability (CIN) is defined as the condition in which cells are unable to properly segregate whole chromosomes or are prone to their structural rearrangements. The immediate consequence of CIN is aneuploidy, a condition in which the number of chromosomes in a cell is not an exact multiple of the haploid set. Notwithstanding, alterations in DNA repair pathways or in the maintenance of telomeres have been suggested as CIN inducers (Thompson & Compton, 2010). A significant number of studies have pointed to abnormalities in the regulation of mitosis as a contributing cause of chromosomal instability (Albertson et al., 2003; Holland and Cleveland, 2009; Pérez de Castro et al., 2007; Schvartzman et al., 2010). Since correct chromosome segregation is essential to maintain an intact genome, errors in this process can lead to mitotic aberrations. Thus, defects in mitotic checkpoints (Cahill et al., 1998), the anchoring of chromosomes to the mitotic spindle (Bakhoum et al., 2009a; Bakhoum et al., 2009b), chromosome cohesion (Zhang et al., 2008b) or the number of centrosomes or cytokinesis (Silkworth and Cimini, 2012), might cause chromosome mis-segregation. This process can be produced in the form of merotelically attached lagging chromosomes, which lag behind at the spindle equator while all the other chromosomes move towards the spindle poles (Cimini et al., 2001). This can result in an aberrant cell division leading to aneuploid daughter cells (Figure 5).

CIN is a hallmark of many tumour types. Indeed it has been proposed as a driving force in tumour initiation (Schvartzman et al., 2010; Shih et al., 2001), as it will be latter explained.

4.2. Aneuploidy

Errors in chromosome segregation can result in daughter cells with an incorrect number of chromosomes that is different from the haploid set, which is called aneuploidy. It is important to note that aneuploidy and CIN are not equivalent. Aneuploidy defines the state of having an abnormal chromosome number, while CIN refers to an elevated rate of chromosome gain or loss. Most human cancers contain aneuploid cells (Albertson et al., 2003) (Pérez de Castro et al., 2007) and since this feature was first postulated by Boveri a century ago (Boveri, 1902), many efforts have been done to know how it contributes to tumourigenesis. Both are common characteristics in tumour development (Schvartzman et al., 2010), however, CIN can generate aneuploidies whereas not all aneuploid cells present CIN and are capable of maintaining an invariable karyotype for a long time.

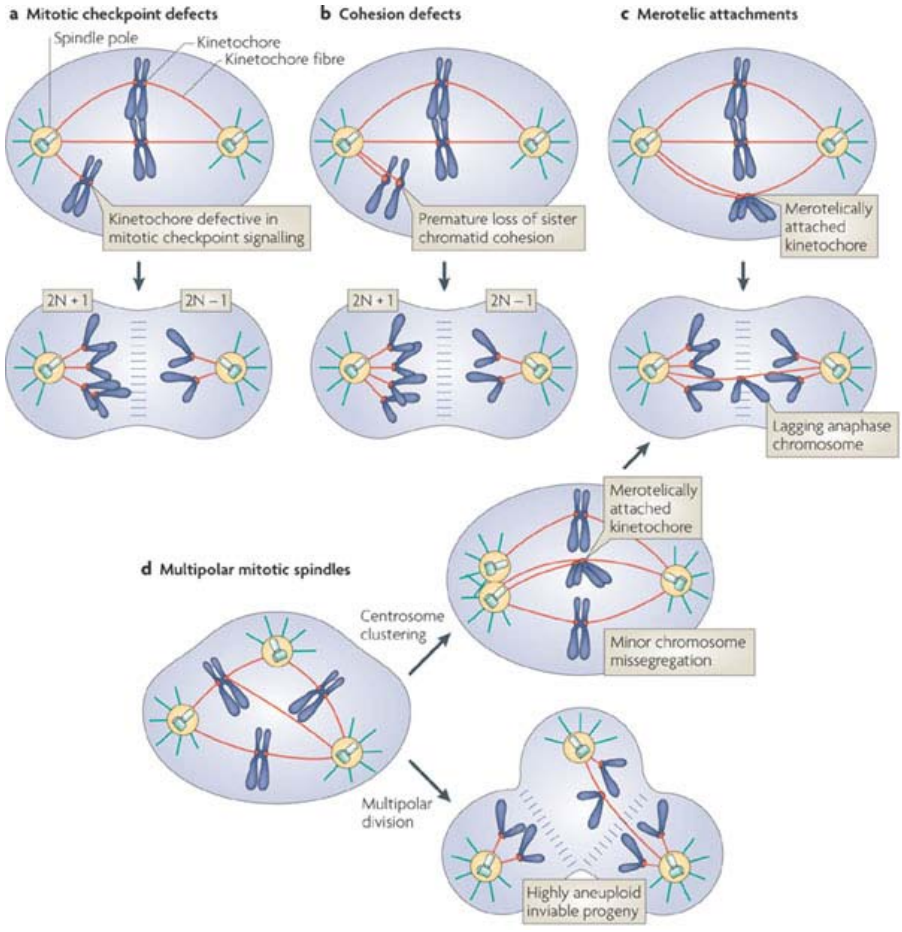


Figure 5. Pathways that lead to aneuploidy .There are different pathways by which cells may gain or lose chromosomes during mitosis. (a) Mitotic checkpoint defects. A weakend mitotic checkpoint might allow cells to enter anaphase in the presence of unattached or misaligned chromosomes. (b) Cohesion defects. Chromosomes can be missegregated when sister chromatid cohesion is lost prematurely or persists during anaphase. (c) Merotelic attachment that occurs when one kinetochore attach to microtubules from both poles of the spindle. If these attachments persist then lagging chromatids are formed that can be misegregated or excluded from daughter cells during cytokinesis. (d) Multipolar divisions are produced when cell possess more than two centrosomes thus forming multiple spindle poles during mitosis.If the defect is not corrected then multipolar division occurs resulting in aneuploid cells Adapted from (Holland and Cleveland, 2009).

Whether aneuploidy alone is a sufficient driving cause during tumourigenesis or rather a mere consequence has been a matter of scientific debate. This clue has remained untested due to the difficulty of selectively generating aneuploidy. It has been described that when depleting the centromere-linked motor protein CENP-E, an increased rate of aneuploidy does drive an elevated level of spontaneous lymphomas and lung tumours in aged animals (Weaver et al., 2007). However, reduction of CENP-E actually inhibits tumourigenesis in the presence of additional genetic damage and in examples of chemically or genetically induced tumour formation, an increased rate of aneuploidy is a more effective inhibitor than initiator of tumourigenesis (Weaver et al., 2007).

It was postulated that aneuploidy drives tumourigenesis by a mechanism in which oncogenes are gained or tumour suppressor genes are lost (Lengauer et al., 1998). More recently it has been proposed that aneuploidy also produces metabolic and energetic alterations that induce cellular imbalances and proteotoxic stress (Torres EM *et al.*, 2007).

At the moment, it is still not known whether CIN promotes or inhibits tumourigenesis or if it contributes to both processes. When excessive DNA damage occurs, CIN probably has a tumour suppressive effect whereas when CIN is moderate alone or in conjunction with other tumoural signals it can contribute to the tumoural process (Figure 6) (Weaver and Cleveland, 2009).

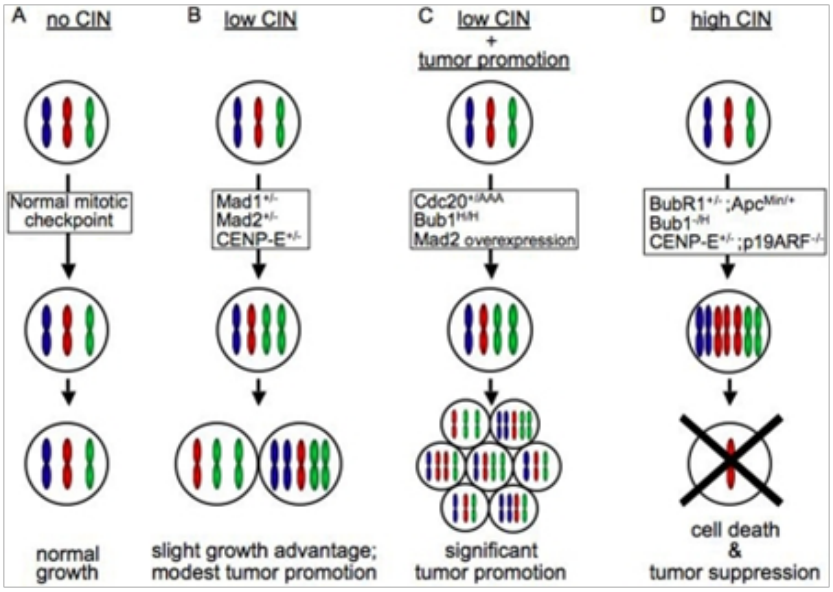


Figure 6.The consequences of CIN. Divisions in a hypothetical cell with three chromosomes are represented. (a) Normal cells do not exhibit CIN and produce genetically identical progeny. (b) Animals heterozygous for the mitotic checkpoint components Mad1, Mad2, or CENP-E missegregate one or a few chromosomes per division (low CIN) and exhibit a modest tumour phenotype. Approximately 20–25% of these animals develop late onset spontaneous tumours. (c) Coupling a low rate of CIN with another tumour-promoting activity, results in a higher rate of tumour formation with a shorter latency. (d) High rates of CIN lead to massive chromosome missegregation and cell death. Adapted from (Weaver and Cleveland, 2009).

4.3. Mouse models of aneuploidy

Several animal models with mutations in genes implicated in the mitotic machinery develop tumours, suggesting a causal relationship between mitotic defects and tumour development (Schvartzman et al., 2010). The main mechanisms behind this state are defects in mitotic checkpoint signalling, in genes implicated in chromosome cohesion, attachment (mainly merotely) or defects on spindle formation (Holland and Cleveland, 2009).

Reduced expression of mitotic components, as well as its overactivation, is associated with an increase of spontaneous cancer and in some cases with aneuploidy. Several mouse models of mitotic regulators have been generated with this purpose, including mitotic checkpoint proteins such as Mad2 or BubR1, mitotic kinases like AurkA or Plk1, APC proteins such as Cdc20 or Cdh1 or motor proteins like CenpE or Hec1. For instance, Bub1 hypomorphic mice develop lymphomas, lung and liver tumours with high incidence (Jeganathan et al., 2007); whereas heterozygous animals for Mad2 develop benign lung tumours with long latencies (Dobles et al., 2000). Likewise, CenpE heterozygous animals evolve benign lung tumours but also splenic lymphomas (Weaver et al., 2007) and heterozygous BubR1 mice are prone to develop colon adenocarcinomas when carcinogenesis is induced (Dai et al., 2004) or in a background of an APC (min) mutation (Rao et al., 2005).

However, whereas mutations that inactivate mitotic genes are rarely observed in human cancers, overactivation of these genes is a much more frequent event than their loss or partial loss of function. In this way, Mad2, Hec1 or Bub1 overexpression that correlate with tumour grade and prognosis in a variety of human tumours (Tanaka *et al*.; 2008; Garber et al., 2001; Shigeishi *et al.,* 2001) is known to drive aneuploidy and to initiate tumourigenesis in inducible murine models (Sotillo *et al.;* 2008; Díaz-Rodríguez *et al.;* 2008; Ricke *et al.;* 2011). These overexpression models result in aneuploidies *in vitro* and in an increase of tumour incidence *in vivo*. Although there is no direct correlation between the level of aneuploidy and the incidence of spontaneous tumours (Jenagathan *et al.;* 2007; Baker *et al.;* 2006; Jenagathan *et al.;* 2005), aneuploidy can increase the risk of neoplastic transformation.

5. The Aurora family of protein kinases

Early work in *Drosophila* led to the identification of Aurora mutants, which carry a loss-of function mutation in a serine/threonine kinase essential for centrosome separation and the formation of bipolar spindles (Glover et al., 1995). The Aurora family of Ser/Thr kinases (*Aurk*) is a highly conserved family of cell cycle regulators which are key to ensure a proper segregation of chromosomes during mitosis. A single Aurora protein exists in budding (*Ipl1*) or fission (*Ark1*) yeast, whereas two family members, Aurora A and Aurora B, are present in worms, flies and frogs. In mammals, there are three different members of this family known as Aurora A, B and C.

5.1. Structure, function, localization and regulation

The three kinases present high structural similarity, 77% between Aurora A and B and 85% between Aurora B and C (Carmena and Earnshaw, 2003). Indeed, Aurora B and C are close paralogs that probably arose from a cold-blooded vertebrate common ancestor (Brown et al., 2004).Although they are highly conserved at the protein level, the three kinases have very distinct functions and localization patterns during cell division (Nigg, 2001) (Figure 7), though all three kinases are involved in the control of many processes required for mitosis. Aurora A and B are ubiquitously expressed in mammals, however, much less is known about Aurora C, whose expression is largely limited to germ cells (Carmena and Earnshaw, 2003). The kinase activity of the Aurora family is regulated by phosphorylation and dephosphorylation processes and their protein levels are controlled by ubiquitin mediated proteolysis.

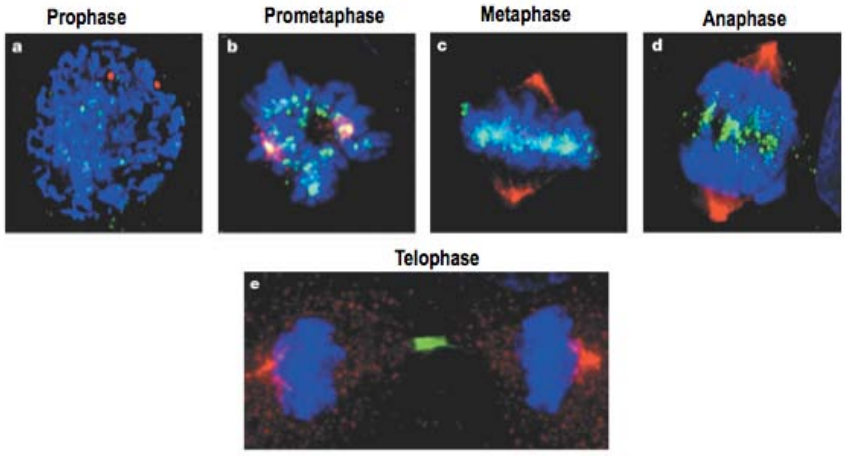


Figure 7. Mammalian Aurora kinases localization. Subcellular localization of Aurora-A (red) and Aurora-B (green) relative to the chromosomes (blue) during (a) prophase, (b) prometaphase, (c) metaphase, (d) anaphase and (e) telophase. During prophase, Aurora-A localizes to the centrosomes, whereas in later stages of mitosis it is at the spindle poles (c, d and e) and also extends up the spindle. During prometaphase and metaphase (a and b), Aurora-B localizes to the centromeres. After anaphase (d), however, Aurora-B localizes to the spindle midzone, and finally accumulates at the midbody during telophase (e). Chromosomes in blue, Aurora A in red and Aurora B in green. Modified from (Carmena and Earnshaw, 2003; Keen and Taylor, 2004).

5.1.1. Aurora A: centrosomes and spindle

Aurora A, the orthologue to the original *Drosophila* kinase localizes on duplicated centrosomes from the end of S-phase to the beginning of G1 phase. Aurora A is required for building a bipolar spindle and for regulating centrosome maturation and separation (by recruitment of proteins involved in microtubule nucleation). It is also involved in mitotic entry and microtubule dynamics (the kinase phosphorylates motor proteins and proteins for astral microtubule nucleation) (Giet et al., 2005) (Barr and Gergely, 2007). Aurora A is implicated in these processes through interaction with its effectors including Tpx2, Eg5, Ndel1 and Lats to name a few.

Aurora A activation depends on its autophosphorylation in the T-loop (Cheeseman et al., 2002) whereas it is negatively regulated by the phosphatase PP1 (Francisco et al., 1994). But its main regulator is Tpx2 (Kufer et al., 2002). When Tpx2 binds to Aurora A it induces a conformational change in a way that the phosphorylated T-loop adopts a compact position providing the platform for substrate binding and hiding the phosphoryl group from the attack by PP1(Bayliss et al., 2003). Moreover, Tpx2 promotes Aurora A autophosphorylation, thus indirectly regulating Aurora A activity (Eyers et al., 2003). It has also been demonstrated that Ajuba and Pak 1 regulate Aurora A activity (Hirota et al., 2003). During mitotic exit, Aurora A is degraded by the proteasome in an APC/C-Cdh1 manner (Castro et al., 2002) in a tightly regulated way since D-box degradation motif is only functional when A-box is non-phosphorylated (Littlepage and Ruderman, 2002).

5.1.2. Aurora B: the catalytic member of chromosomal passenger complex (CPC)

Aurora B, was first identified in *S. cerevisiae* (as Ipl1) in a screen for mutants that display an increase in ploidy (Chan and Botstein, 1993). Aurora B is the catalytic component of a multiprotein complex, named the Chromosomal Passenger Complex (CPC), which comprises other three non-enzymatic subunits: the scaffolding protein Incenp, Survivin and Borealin/DasraB (Ruchaud et al., 2007), that controls the targeting, activity and stability of Aurora B, and play key functions during mitosis including chromosome interactions with microtubules, chromatid cohesion, spindle stability and cytokinesis (reviewed in (Carmena et al., 2009) (Figure 8).

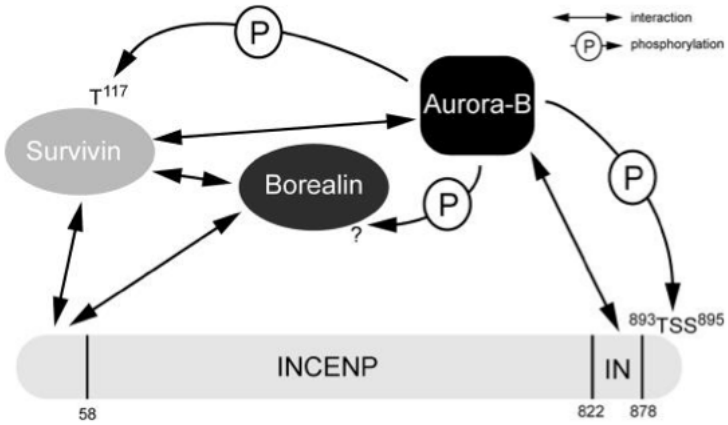


Figure 8. Schematic representation of the Chromosomal Passenger Complex (CPC). Schematic representation of direct interactions between CPC proteins and phosphorylations of Aurora-B within the CPC. Survivin and Borealin interact with the NH_2 terminus of INCENP, whereas Aurora-B binds the COOH-terminal IN-box in INCENP. Mapped Aurora-B phosphorylation sites are indicated (Vader et al., 2006).

The CPC is highly dynamic, it is firstly located on chromosome arms and in prometaphase and metaphase it is concentrated in inner centromeres. In anaphase, it relocates to the central spindle and during telophase and cytokinesis it relocates to the midbody. The CPC is one of the upstream regulators of kinetochore-microtubule attachment by recruiting to the kinetochore a growing number of proteins including: inner centromeric proteins (Sgo1, Sgo2, MCAK), microtubule-kinetochore regulators (Ndc80, CenpE, Plk1) or SAC proteins such as Mad2, BubR1 or Mps1 (Kelly and Funabiki, 2009). Some of these molecules are Aurora B substrates such as the mitotic centromere associated kinesin (MCAK) and the kinesin family member Kif2b (Nezi and Musacchio, 2009)., or Hec1/Ndc80, component of the KMN network (named for the Knl1 , the Mis12 and the Ndc80 complex; is part of the protein architecture within kinetochores that links centromeric DNA to the plus ends of spindle microtubules (Kline-Smith et al., 2004). By their recruitment to outer kinetochores, Aurora B promotes the correction of microtubule mis-attachments (Cheeseman and Desai, 2008; Santaguida and Musacchio, 2009; Maresca and Salmon, 2010).

It has been recently described that Aurora B substrate phosphorylation depends on the distance of Aurora B to the substrate on the inner centromere. So that when Aurora B is recruited to the kinetochores it prevents inappropriate attachments and activates the SAC (Liu et al., 2009), thus promoting chromosome bi-orientation by correcting mis-attachments. In response to a lack of tension in budding yeast Aurora kinase, Ipl1 is required for SAC function (Biggins and Murray, 2001), although in mammals its role in SAC functioning is on debate (Ditchfield et al., 2003; Hauf et al., 2003; Santaguida et al., 2011). Likely, mammalian Aurora B is not essential for the SAC but it might partially contribute as a separate arm (Morrow et al., 2005) to the SAC response by recruiting BubR1 to kinetochores (Fernández-Miranda et al., 2011). This was first proposed as a result of studies in budding yeast suggesting that Aurora activity was required for kinetochores to release bound microtubules (Tanaka et al., 2002). Aurora B in the centromere would continually promote disruption of kinetochore–microtubule attachments until the bi-oriented chromosome came under tension.

Aurora B is specifically required for the correction, before anaphase, of merotelic attachment (a single kinetochore is attached to microtubules emanating from both spindle poles), an error of kinetochore-microtubule attachment that occurs frequently during the early stages of mitosis but is not detected by the spindle assembly checkpoint (SAC) (Gregan et al., 2011; Lampson and Cheeseman, 2011). These attachments can result in improper chromosome segregation and are a significant source of aneuploidy (Cimini et al., 2001; Cimini et al., 2002). This correction is likely possible by the recruitment of MCAK that is capable of depolymerizing improper attach microtubules (Andrews et al., 2004) (Lan et al., 2004) (Zhang et al., 2007) together with additional proteins, thus allowing reorientation of the kinetochore towards the correct spindle pole (Figure 9).

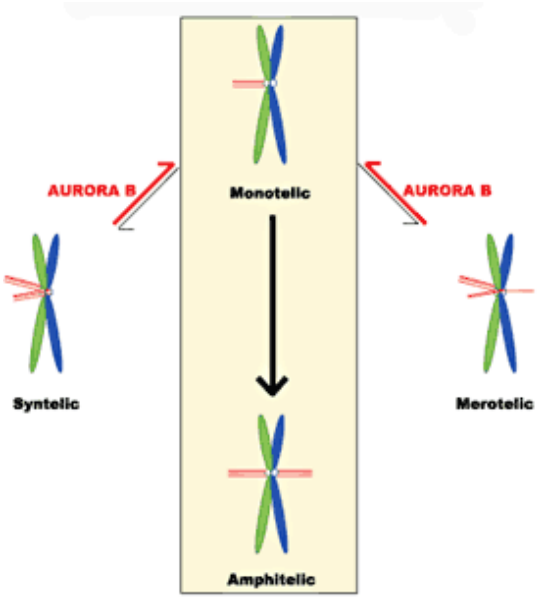


Figure 9. Aurora B promotes chromosome biorentation on the mitotic spindle.The first attachement of chromosomes to spindle microtubules is monotelic (one kinetochore bound and one kinetochore). When both kinetochores are attached to the same spindle pole, syntelic attachment is formed or it can occur that a single kinetochore is bound to microtubules from both poles thus generating merotelic attamentes. Aurora B, in the inner centromere promotes the formation of monotelic attachments. Eventually, chromosomes become attached to both spindle poles (amphitelic attachment). Modified from (Montembault and Pringent, 2005).

Aurora B and the CPC are also required for stability of the bipolar mitotic spindle (Adams et al., 2001). Furthermore, several studies have shown that Aurora B participates in the control of sister chromatid cohesion (Losada et al., 2002) by regulating the association of separase with mitotic chromosomes (Yuan et al., 2009). During anaphase, Aurora B concentrates at the spindle midzone and equatorial cortex, accumulating ultimately at the midbody where together with all of the CPC components plays essential roles in cytokinesis (Terada et al., 1998).. Aurora B is also a key regulator of abscission timing if unsegregated chromatin is trapped at the furrow ingression site in human cells (Steigemann et al., 2009)

Moreover, Aurora B is a histone kinase that phosphorylates in mitosis histone3 at Ser10 and probably 28 and the serine 7 in CENP-A (a modified histone H3 that determine where kinetochores are assembled) that seems to be necessary for chromosome condensation and segregation (Giet and Glover, 2001) (Nowak and Corces, 2004; Prigent and Dimitrov, 2003) (Johansen and Johansen, 2006). Aurora B is also implicated in spindle stability and during cytokinesis. Its local inactivation is necessary for completion of abscission (Zeitlin et al., 2001) (Goto et al., 2003) (Guse et al., 2005) (Steigemann *et al*., 2009).

Aurora B is mainly regulated by the interaction with the other CPC components: Incenp, Survivin and Borealin. All the CPC members are physically and functionally dependent in a way that when knocking down any of the members it delocalizes the others, thus impairing a correct mitotic progression (Gassmann et al., 2004; Jeyaprakash et al., 2007).

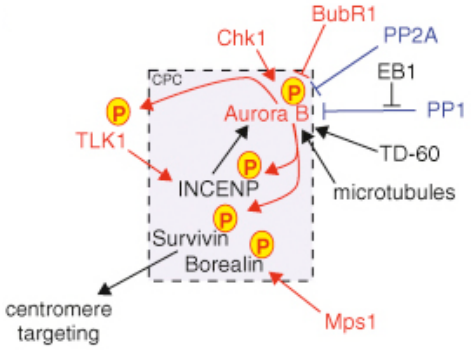


Figure 10. Aurora B regulation. The major regulators of Aurora B kinase. Protein kinases are indicated in red and phosphorylation events by red arrows. Protein phosphatases are indicated in blue. Adapted from (Carmena et al., 2009).

INCENP binding to Aurora B increases basal activation of the kinase via its T-loop, which achieves full activity by autophosphorylation in a positive feedback loop (Bishop and Schumacher, 2002) (Honda et al., 2003). Borealin/Dasra B is suggested to promote local clustering that leads to Aurora B auto-activation at the centromere (Sessa et al., 2005) (Kelly et al., 2007). Moreover, phosphorylation of Borealin by the checkpoint kinase Mps1 leads to an increased activation of the kinase at the centromere which is known to be essential for Aurora B activity and chromosome alignment (Jelluma et al., 2008) (Figure 10). Survivin appears to be involved in stimulating Aurora B activity and helps to target Aurora B to its substrates (Bolton et al., 2002) (Honda et al., 2003) (Chen et al, 2003).

Phosphatases negatively regulate the activity of the kinase. It is known that protein phosphatase 1 (PP1, a ubiquitos serine/threonine phosphatase) and the protein phosphatase 2 (PP2A) are the major counteracting phosphatases of Aurora B (Emanuele et al., 2008; Francisco and Chan, 1994) (Sugiyama et al., 2002; Sun et al., 2008). In vertebrates at least six different PP1holoenzymes counteract Aurora B signaling at the kinetochore. These phosphatases oppose the recruitment (PP1-Mypt1) or retention (PP1α and PP1γ/Repoman, localized at the outer kinetochore are able to remove Aurora B phosphorylation marks) of Aurora B at the inner centromeres (Trinkle-Mulcahy et al., 2006), or promote the inactivation of the kinetochore-associated Aurora B (PP1/Sds22), and dephosphorylation of Aurora B substrates (PP1/KNL1, PP1/CENP-E and PP1/KIF18A) (Figure 10).

During metaphase, when sister chromatids come under tension and PP1 holoenzymes dephosphorylate Aurora B thus reversing Aurora B signaling and triggering spindle checkpoint silencing (Lesage et al., 2011; Qian et al., 2011). The checkpoint protein BubR1 inhibits Aurora B activity at the kinetochore to promote the formation of stable microtubule–kinetochore attachments (Lampson and Kapoor, 2005). In anaphase, binding of the microtubule plus-end-binding protein EB1 shields the kinase T-loop from PP2A dephosphorylation (Sun et al., 2008). Additionally, INCENP dephosphorylation in budding yeast by Cdc14 phosphatase upon separase activation has been shown to be important for the transfer of the CPC to the central spindle (Pereira and Schiebel, 2003). Indeed, the dephosphorylation of the CPC and Aurora B substrates is crucial for completion of mitosis (Lesage et al., 2011). More specific regulations of Aurora B are shown in Figure 10.

In terms of Aurora B degradation, the kinase is regulated by ubiquitin posttranslational modifications. During early mitosis, Aurora B is targeted by a Cul3-containing Scf ubiquitin ligase in order to remove a fraction of Aurora B from mitotic chromosome allowing its accumulation on the central spindle during anaphase (Sumara et al., 2007). Then, during anaphase Aurora B requires the action of APC/C-Cdh1 to translocate to the spindle midzone (Floyd et al., 2008). After completion of cytokinesis, the remaining pool of Aurora B is targeted for degradation by APC/C-Cdh1 (Floyd et al., 2008; García-Higuera et al., 2008). Aurora B proteolysis does not depend on its D-boxes (RXXL), but it does require KEN boxes and A-boxes (QRVL) located within the first 65 amino acids (Nguyen et al., 2005).

5.1.3. Aurora C: the unknown member of the family

Whereas Aurora A and B are very well known kinases and ubiquitously expressed in mammals, much less is known about Aurora, whose expression is largely limited to germ cells (Carmena and Earnshaw, 2003) and at low levels to thyroid and other cell types (Lin et al., 2006) (Ulisse et al., 2006). Aurora C is known to have a specific role in spermatogenesis (Yanai et al., 1997), (Tang et al., 2006) (Dieterich et al., 2007b; Kimmins et al., 2007b) and in oogenesis (Sharif et al., 2010) but its role in mitosis is still unresolved.

In terms of its structure, Aurora C is a close paralogue of Aurora B, most probably both proteins arose from a relatively recent common ancestor (Brown *et al*; 2004), in fact, Aurora C has a localization pattern similar to Aurora B along mitosis (Dutertre et al., 2005) and can bind members of the CPC complex, like Aurora B (Li et al., 2004). Moreover, Aurora C ectopic expression can rescue Aurora B loss of function and like other Auroras is activated by some of its substrates, in particular by INCENP (Sasai et al., 2004) (Yan et al., 2005) (Slattery *et al*, 2009). Recently our group described that Aurora C can compensate for the lack of Aurora B in somatic cells (Fernández-Miranda et al., 2011). However, in interphase Aurora C colocalizes with Aurora A (Dutertre et al., 2005). On the other hand, our group also described that Aurora C is the kinase responsible for CPC function during the first embryo divisions (pre-implantation stage) (Fernández-Miranda et al., 2011). Strikingly, nobody has yet localized the endogenous protein nor analysed whether the kinase is expressed in normal somatic cells and what its function would be. Additional roles for Aurora C in somatic cells would include non-mitotic functions such as gene regulation via histone 3 phosphorylation (Price et al., 2009).

How Aurora C is regulated is not known so far, but like other Auroras is activated by some of its substrates, in particular by association with INCENP (Li et al., 2004; Sasai et al., 2004). Due to the lack of KEN and A-boxes motifs, Aurora C is less prone to degradation (Schindler et al., 2012) as was previously observed in Hela cells (Sasai et al., 2004).

5.2. Mouse models of Aurora kinases

Different mouse models have been generated for the study of Aurora A. In some of them *Aurka* has been genetically ablated (Cowley et al., 2009; Lu et al., 2008; Pérez de Castro et al., 2013; Sasai et al., 2008) and its disruption leads to embryonic lethality (E 3.5) due to severe defects in the mitotic spindle. It has also been recently described its role in tissue regeneration and tumour development in adult mammals (Pérez de Castro et al., 2013). On the other hand, several mouse models have been generated to study the effects of its overexpression in different tissues. The last published studies postulate that overexpression of Aurora A leads to mammary tumour formation at a low frequency and after a long latency and it also contributes to laryngea squasmous cancer progression (Zhang et al., 2008a) (Wang et al., 2006). The tumourigenesis associated with Aurora A overexpression is secondary to genetic instability that is characterized by centrosome amplification, tetraploidization and PMSC and is also associated with an activation of AKT (Bischoff et al., 1998; Zhang et al., 2012).

The function of Aurora B *in vivo* has been poorly studied using mouse models. Its role in spermatogenesis has been analysed in a mouse model confirming the important role of Aurora B during male meiosis (Kimmins et al., 2007b). Recently, our group has generated a conditional knock-out model for Aurora B that lead to the discovery of its dispensability during early embryonic development and that uncovers and essential role of Aurora C during this stage (Fernández-Miranda et al., 2011). However, the effect of Aurora B depletion in adult mammals has not been analysed so far. Moreover, due to the high correlation between the expression of Aurora B and the tumourigenicity of several human tumours (see Table 1) the study of its overexpression *in vivo* is crucial to understand the cause of the disease and no model to date has been described.

Regarding Aurora C, a knock-out model for this regulator was generated (Kimmins et al., 2007b). The mice were viable and normal but male were infertile, which suggested a role for the kinase in spermatogenesis. A recent study in oocytes describes that Aurora C is recruited during oocyte maturation. The posttranscriptional regulation of *Aurkc* mRNA, coupled with the greater stability of Aurora C protein, provides a means to ensure sufficient Aurora kinase activity, despite loss of Aurora B, to support both meiotic and early embryonic cell divisions (Schindler et al., 2012). However, why germs cells express Aurora C is unclear.

5.3. Aurora kinases and cancer

For tumour formation, cells must acquire a series of genetic alterations that will promote its transformation in malignant cells. Several human tumoural analysis and studies in animal models have linked the similarity between tumoural formation and Darwin's evolutive theory. In both cases, successive genetic alterations provide a growth advantage that in case of tumours produces the transformation of a normal cell into a

tumoural one. Tumoural cells not only acquire more proliferative capacity but also the capacity to avoid cell death signals, a non-limited potential to duplicate its genetic material, they develop angiogenic capacities and also mechanisms to invade adjacent tissues (Hanahan and Weinberg, 2011; Luo et al., 2009).

Several evidences relate Aurora kinases with tumoural progression and malignant cell transformation. The first studies that link Aurora kinases with tumourigenesis came from the observed overexpression of Aurora A and B in primary breast (Sen et al., 1997) and colon samples (Bischoff et al., 1998; Sen et al., 1997). *Aurka* is located on human chromosome 20q12, a hotspot of amplification in tumours that is also associated with poor prognosis in patients (Sen et al., 1997). Subsequent studies identified other human malignant cancers in which *Aurka* is known to be overexpressed/amplified (Table 1) and this overexpression has been linked to chromosomal instability (CIN), one of the major drivers of the aneuploid state (Perez de Castro et al., 2007; Perez de Castro et al., 2008) (Miyoshi et al., 2001; Tanaka et al., 1999). Aurora A, has long been claimed to function as an oncogene *in vitro* (Bischoff et al., 1998) and *in vivo* by inducing mitotic abnormalities (Zhang et al., 2004).

The role of Aurora B in tumour induction is less clear, although some evidences point to a role for the protein in tumourigenesis. In 2006, a computational method that correlates CIN in human tumours with several genes expression was developed and *Aurka/b* were within the CIN70 gene expression signatures associated with high chromosome instability (CIN) in human cancers and both genes presented a high correlation between their level of expression and the severity of the disease (Carter et al., 2006). Moreover, *Aurkb*, as *Aurka*, is amplified in primary human tumours where elevated levels of the Aurora B transcript or protein are often associated with poor clinical prognosis and correlate with genetic instability and metastasis grade (Table 1). Some *in vitro* studies have demonstrated that forced expression of Aurora B can enhance Ras-induced cell transformation (Kanda et al., 2005). A number of studies in *in vitro* or xenografts experiments have shown that Aurora B overexpression induces aneuploidy and increases invasiveness, suggesting its role as promoter of metastasis (Ota et al., 2002; Tatsuka et al., 1998; Terada et al., 1998) (Steigemann et al., 2009). Furthermore, several studies suggest that tetraploidy is an important mediator of Aurora B induced tumourigenesis (Nguyen et al., 2009). In a similar manner, it has been hypothesized that Aurora B is a critical target through which overexpressed Bub1 drives aneuploidization and tumourigenesis (Ricke RM *et al.,* 2011). However, the causal connection between *Aurkb* abnormal expression, aneuploidy and cancer has not been proved so far.

Little is known about the third member of Aurora kinase family. In normal physiological conditions Aurora C is expressed only in testis (Hu et al., 2000). However, Aurora C is also overexpressed in cancer cell lines (Kimura et al., 1999), where its expression is correlated with the aggressiveness of the tumour (Table 1). The first evidence of its overexpression was observed in thyroid tumours (Ulisse et al., 2006). *In vitro*, overexpression of AuroraC gives rise to polyploid cells. And like for the other Aurora kinases, the phenotype is aggravated in the absence of p53 (Dutertre et al., 2005).

Aurora kinases are relevant cancer targets in the clinic. Several inhibitors have been developed (Taylor and Peters, 2008) (Malumbres and Perez de Castro, 2014). Most aurora kinase inhibitors are small molecule compounds designed to bind to the ATP-binding pocket in a competitive and reversible manner. Around 10 pan-Aurora inhibitors have been reported in clinical trials or preclinical stages for cancer treatment (Giles et al, 2013, Foran, 2014). The first and best described compounds were Hesperadin, VX-680 and ZM447439 (Ditchfield et al., 2003; Harrington et al., 2004; Hauf et al., 2003). These three compounds produce cytokinesis failure and and polyploidy. Treatment of mice with VX-680 efficiently reduces tumour growth in xenograft mice with established tumours, also induces apoptosis and reduces histone H3 phosphorylation (Harrington et al., 2004). More recently, Danusertib and AT9283 are being used in the clinic as Aurora pan inhibitors (phase I and II clinical trials) (Meulenbeld et al, 2012, Dent et al, 2013). Particularly, several Aurkb selective inhibitors have recently being used in the clinic (phase I, II and III): BI811283, AZD1152, GSK1070916 (Malumbres and Perez de Castro, 2014).

Table 1. Human cancers with alterations in Aurora kinases expression levels.

AURORA KINASE A

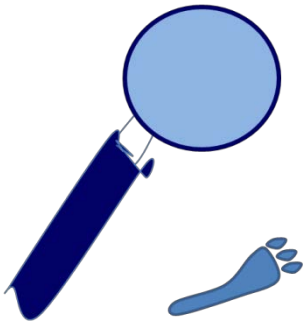
Type of cancer	Alteration	Reference
Breast	Overexpression	(Miyoshi et al., 2001; Tanaka et al., 1999)
Colon	Overexpression	(Bischoff et al., 1998; Katayama et al., 1999; Sen et al., 1997)
Pancreas	Overexpression	(Li et al., 2003)
Ovary	Overexpression	(Do et al., 2014)
Bladder	Urinary prognostic marker	(de Martino et al., 2014; Lei et al., 2011; Sakakura et al., 2001)
Neuro-blastoma	Overexpression	(Zhou et al., 1998)

AURORA KINASE B

Type of cancer	Alteration	Reference
Colon	Overexpression/ correlation	(Katayama et al., 1999)
Astrocytomas	Expression/ correlation	(Araki et al., 2004)
Seminomas	Correlation	(Chieffi et al., 2004)
Non-small lung carcinoma	Genetic instability correlation	(Smith et al., 2005)
Prostate	Malignancy/ proliferation correlation	(Chieffi et al., 2006)
Thyroid	Overexpression correlation	(Sorrentino et al., 2005)
Lymph node	Invasion	(Vischioni et al., 2006)
Breast	Elevated expression	(Hegyi et al., 2012)
Head/neck SCC	Prognostic factor	(Pannone et al., 2011; Qi et al., 2010)
Hepatocellular carcinoma	Predictive factor for recurrence	(Tanaka et al., 2008)
Mammary epitelial tumour	Overexpression in nude mice	(Nguyen et al., 2009)

AURORA KINASE C

Type of cancer	Alteration	Reference
Thyroid	Overexpression	(Ulisse et al., 2006) (Wiseman et al., 2007)
Breast	Overexpression	(Zekri et al., 2012)
Prostate	Gene amplification Overexpresion biomarker	(Dhanasekaran et al Zekri et al., 2012)



Objectives/ Objetivos

Objectives

The aim of the work presented in this thesis was to study the *in vivo* consequences of Aurora B/C deregulation in mammals. This work has been focused on the study of the functional characterization caused by Aurora B depletion or overexpression by using conditional mouse models, as well as on analysing the role of Aurora C, the unkown member of the family, in stemness induction. With this purpose the following objectives have been proposed:

1. Understand the *in vivo* effects of Aurora B depletion in adult mammals.

2. Study the physiological outcomes of Aurora B overexpression in culture cells and adult mammals.

3. Explore whether the tumour suppressor function of the p53 pathway is compromised by the overexpession of Aurora B.

4. Determine the role of Aurora B and C in pluripotency.

Objetivos

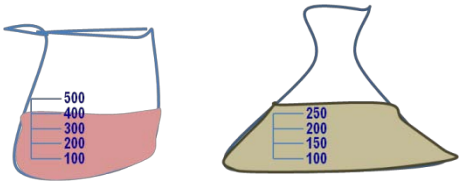
El principal objetivo del trabajo presentado en esta tesis fue estudiar las consecuencias de la desregulación de Aurora B/C en mamíferos. Este estudio se ha centrado en la caracterización funcional causada por la depleción/sobreexpresión de Aurora B utilzando modelos condicionales de ratón, así como el análisis del papel de Aurora C, miembro desconocido de la familia, en la inducción de la condición pluripotente. A partir de este objetivo principal se han propuesto los siguientes objectivos:

1. Comprender los efectos de la eliminación de Aurora B en mamíferos adultos.

2. Estudiar las consecuencias fisiológicas de la sobreexpresión de Aurora B en células en cultivo y mamíferos adultos.

3. Investigar si la function del supresor tumoral p53 está comprometida por la sobreexpresión de Aurora B.

4. Determinar el papel de Aurora B and C en pluripotencia.



Materials and Methods

Material and Methods

1. Genetically modified mouse models

1.1. Animal housing

Mice were housed in the pathogen-free animal facility of the Centro Nacional de Investigaciones Oncológicas (CNIO, Madrid) following the animal care standards of the institution. These animals were observed daily and sick mice were euthanized humanely in accordance with the Guidelines for Humane End Points for Animals used in biomedical research. All animal protocols were approved by the ISCIII Committee for animal care and research. Mice were maintained in a mixed 129/Sv (25%) x CD1 (25%) x C57BL/6J (50%) background).

1.2. Generation of mouse models

1.2.1. Construction of targeting vectors

A conditional loss-of-function model of *Aurkb* was generated in our laboratory (Fernández-Miranda et al., 2011). The knock-in targeting vector that gives inducible expression of Aurora B gene under a minimal tetracycline promoter was constructed by Gonzalo Fernández-Miranda in our lab in collaboration with Earnshaw's lab using a modified version of Ptre-tight vector (Clontech). This modified Ptre-tight vector contains the puromycin gene sequences driven by the β-actin promoter for positive selection of the clones. Genomic sequences of 5' and 3' homology arms were amplified by PCR from previous BACs and cloned in PGEM-T (Promega) intermediate vector. The cassette PGK-TK was amplified from Ppnt vector and was used for negative selection of the clones. The 5' arm was then cloned in Ptre modified vector using *AvrII/SpeI* restriction sites, the 3'arm was cloned in *PvuII/NotI* and the PGK-TK cassette in *ClaI/ClaI* (Figure 12).

1.2.2. Generation of Aurora B conditional, knock-in and null alleles

To facilitate homologous recombination, mouse ES cells V6.4 obtained from a hybrid (129 x C57BL/6J) strain were electroporated with 100 μg of linearized DNA from the corresponding targeting vectors. Recombinant ES cells and clones were selected in the presence of G418 (neomycin). This step was done by the Transgenic Unit of the CNIO. The screening of the recombinant clones was performed by Southern blot analysis using new restriction sites from the recombinant alleles and probes external to the homology arms. Positive recombinant clones were either aggregated with morulas CD1 or microinjected into C57BL/6J blastocysts by the Transgenic Unit of the CNIO. The resulting male quimeras were crossed with wild-type females for transmission of the recombinant allele.

Heterozygous recombinant mice *Aurkb*$^{+/loxfrt}$ (conditional knock-out model) were first crossed with TgpCAG-Flpe transgenic mice (Rodríguez et al., 2000) that ubiquitously expressed Flp recombinase, to remove the neo selection marker and thus, to generate the conditional *Aurkb*(lox) allele. To generate the null allele we crossed *Aurkb*$^{+/lox}$ mice with TgCMV-Cre transgenic mice (Zuazua-Villar et al., 2014) that ubiquitously expressed Cre recombinase. In the *Aurkb*(lox) allele, Cre mediated recombination between the two loxP sites excises exons 2-6. By tamoxifen treatment *Aurkb* allele is conditionally depleted.

On the other hand, for the generation of the inducible Aurora B knock-in mouse, the "hijack" promoter strategy was used (Samejima et al., 2008). The conditional targeting construct contained a minimal tetO-CMV promoter (Tet-P) inserted in front of the *Aurkb* ATG, thus replacing the Aurora B endogenous promoter, and a puromycin cassette for positive selection of the clones (Figure 12). In the case of the inducible mice, after homologous recombination (HR) in ES, clones were selected using puromycin resistance. *Aurkb*$^{+/loxtet}$ clones were identified using Southern blot analysis (Figure 12a). These ES cells were aggregated to generate *Aurkb*$^{+/loxtet}$ mice and the puromycin-resistant cassette was removed in vivo by crossing with transgenic mice expressing the *Cre* recombinase resulting in the *Aurkb*^{tet} allele. The Aurora B inducible mice were then crossed with mice expressing the Rosa26-rtTA allele (Beard et al., 2006), which expresses a tetracycline-inducible M2rtTA transactivator driven from the endogenous ubiquitous Rosa26 promoter (*Rosa26*$^{M2rtTA/M2rtTA}$). Addition of tetracycline modifies the configuration and binding of rtTA to the tetO sequences so that rtTA binds to and activates expression of the tet-P (Tet-ON system) (see Figure 13). Tumor latency in all animals has been considered equivalent to lifespan.

i4F (four Yamanaka factors) mice were reported previously and were obtained from M.Serrano's laboratory with the purpose of obtaining mouse embryonic fibroblasts (MEFs) to induce pluripotent cells (Abad et al., 2013).

1.2.3. Mice genotyping

For genotyping alleles for the different mouse models we isolated tail DNA from 3-4 week old mice and we performed a PCR amplification reaction using the oligonucleotides shown in Table 2 using these conditions: 94ºC during 4 minutes followed by 35 cycles of DNA denaturalization at 94ºC during 30 seconds, primer annealing at 60ºC during 30 seconds and polymerase extension at 72ºC during 60 seconds, ending with a single elongation cycle of 7 minutes at 72ºC.

Table 2. Oligonucleotides used for mouse models locus genotyping.

Mouse Model	Allele	Size (bp)	Sequence (5´-3)
Aurkb conditional knockout	*Aurkb*(+)	358	Fw: AGGGCCTAATTGCCTCTTGT
	Aurkb(lox)	491	Rv: GGGCATGAATTCTTGAGTCG
	UbCre(+)	495	Fw:CACCCGTTCTGTTGGCTTAT
	UbCre(T)	390	Rv: ATGTTTAGCTGGCCCAAATG
Aurkb inducible knockin	*Aurkb* (+)	482	Fw: AGTAGTCTCTGCCCCCTGGT
	Aurkb(lox)	430	Rv_1: GAGATGGGTTGGGTAGCAGA
	Aurkb(tet)	368	Rv_2: ATGGGGAGAGTGAAGCAGAA
			Rv_3:CTGATAGGGAGTAAACTCGACA
	Rosa26(+)	600	Fw-:GGAGCGGGAGAAATGGATATG
	Rosa26(KI)	400	Rv1:AAAGTCGCTCTGAGTTGTTAT
			Rv2:GCGAAGAGTTTGTCCTCAACC
4 Yamanaka's factors	*OSKM*(+)	408	Fw: GGATGGAGTGGGACAGAGAA
	OSKM(KI)	300	Rv: GTGCCGATCCGTTCACTAAT

1.2.4. Treatments in live animals

In order to deplete *Aurkb* in the conditional model mice were fed with tamoxifen since weaning (previously treated with an accommodation diet). To specifically remove Aurora B in the skin, wild-type and transgenic mice were anesthesized previous to hair removal (performed as previously described (Ruzankina, 2007) and tamoxifen was applied topically during 6 days. For *Aurkb* induction, mutant and wild type mice (as controls) were fed with doxycycline-supplemented food (Harlan Laboratories Models) since weaning (long treatment).

1.3. Histology

For histological analysis, dissected organs were fixed overnight in 10% buffered formalin (Sigma) and embedded in paraffin wax. Specimens were dehydrated in 70% ethanol and processed by the Histopathology Facility Unit at CNIO. Sections of 3-5 μm thickness were stained with hematoxylin and eosin (H&E). Additional inmunohistochemical examination of the tissues and pathologies was performed using specific antibodies against the antigens described in Table 4. To quantify positive cells in the different organs, paraffin sections stained with antibodies were examined by using an Olympus BX51 microscope equipped with objective lenses (40/0.75, 20/0.4, 10/0.25, and 4/0.1). Images were analyzed by ImageJ software (National Institutes fo Health, Bethesda, Maryland, USA). Total number of cells was counted in each 20X observation field. The pathological analysis was performed with the help of Marta Cañamero and Alba de Martino (Histopathology Unit of the CNIO). Hematopoietic parameters in blood samples were obtained using an Abacus Junior Vet Hematology Counter (Practice CVM).

1.4. In vivo isolation of peripheral lymphocytes and splenocytes

1.4.1. Lymphocytes and splenocytes isolation

Peripheral blood lymphocytes were extracted from wild-type and inducible doxycycline treated mice along the time of treatment. Blood was extracted from the cheek of mice and right away lymphocytes were isolated by means of Lymphocyte-M cell separation medium (Cedarlane). Lymphocyte-M is a density separation medium specifically designed for the isolation of viable lymphocytes from murine lymphoid cell suspensions. Cytospin preparations from these samples (interphasic lymphocytes) were analysed by Chromosome Fluorescent In Situ Hybridization (FISH).

Splenocytes were isolated as follows: Mice were euthanized; spleens were extracted and maintained in PBS. With the help of a cellular sieve (40 μm; Bencton-Dickinson) the spleen was disgregated and homogenized. A total number of 10^7 cells were plated in six well plates to maintain cells at high density. In order to stimulated splenocytes to enter cell cycle, they were cultured in RPMI (Gibco) with the addition of concanavalin A (3 μg/ml; conA, Sigma) and Lipopolysaccharide (25 μg/ml, LPS, Sigma) during 96 hours.

1.4.2. Karyotyping and scoring of aneuploidy

Cultured cells were exposed to colcemid for 5 hours and hypotonically swollen in a 40% full medium, 60% tap water for 5.5 minutes. Hypotonic treatment was stopped by adding an equal volume of Carnoy's solution (75% pure methanol, 25% glacial acetic acid), cells were then spun down and fixed with Carnoy's solution for 10 minutes. After fixation, cells were dropped from a 5-cm height onto glass slides previously treated with 45% of acetic acid. Slides were mounted with ProLong Gold anti-fade reagent with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen), and images were acquired with a Leica D3000 microscope and a 60 PlanApo N 1.42 N.A. objective. The karyotyping was performed by counting the number of chromosomes from 50 cells per genotype using ImageJ software.

Peripheral blood lymphocytes were analyzed by FISH using probes from two mouse BAC clones (a gift from A. Losada, CNIO) of chromosome 8 (RP23-310L10) and chromosome 11 (RP23-263C13) were labeled with SpectrumGreen-dUTP, and SpectrumRed-dUTP (Vysis), respectively. The BAC DNAs were labeled by nick translation (Abbot Inc.) according to standard protocols. FISH probes were denatured by incubation for 5 min at 90C and then applied to dried slides, before a coverslip was added and sealed. Slides were incubated overnight at 37C in a humidified chamber and then washed for 5 min in 50% formamide in SSC 2x and for 3 min in 4x SSC + 0.05% Tween 20, all at 45C. Cells were stained with DAPI and and mounted using Prolong. Image stacks were acquired on a Nikon Eclipse TE 2000-E inverted spinning disk confocal microscope and flattened into a maximal projection before being scored. Both gains and losses of chromosomes were counted.The number of hybridization signals for these probes was assessed in a minimum of 100 interphase nuclei with well-delineated contours. For performing

FISH in tissue sections, paraffin slides (3 µm thick) were pre-treated. First, the slides were immersed in xylene for 2 x 20 min, and then for 1 min each in 100%, 85%, and 70% ethanol. Slides were washed in running tap water and immersed in ddH2O before being pretreated with 0.2 N HCl for 20 min, washed in ddH2O for 3 min, incubated in 8% sodium thiocyanate for 30 min at 80C, washed in 2xSSC for 3 min, and digested in 0.5% pepsin in 0.2 N HCl for 1 hr at 37C. Slides were washed at 37C in ddH2O for 1 min and in 2 x SSC for 5 min and then dehydrated for 1 min each in 70%, 85%, and 100% ethanol. Slides were dried in a 45C oven before being denatured in 70% formamide in 2x SSC at 55 C for 40 s. Denatured slides were placed in ice-cold 70% ethanol and then room temperature 70%, 90%, and 100% ethanol for 3 min each. Slides were then air dried before hibridization with the probes was performed.

In addition, aneuploidy was determined by measuring nuclear volumes in tissues. Interphasic nucleus volumes were calculated using the following algorithm: 4/3_p_r3, where r ¼ is the Feret diameter/2. Feret diameter was calculated using ImageJ on images of DAPI-stained fibroblasts or H&E-stained tissues (Pérez de Castro et al., 2013)

2. Cell Culture

2.1. Mouse embryonic fibroblasts (MEFs)

Mouse embryonic fibroblasts (MEFs) were prepared from E13.5 embryos and cultured using standard protocols (García-Higuera et al., 2008). E13.5 embryos were extracted from the uterus of pregnant females. The placenta was removed and embryos were isolated from the yolk salk. The embryo without the liver and the head was minced, and dispersed in 0.1% trypsin (5 min at 37°C). Cells were grown for two population doublings and then frozen. All cultures were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). MEFs infection was performed during 2 days using adenoviruses expressing histone H3 tagged witht the green fluorescent protein (H3-GFP) obtained from the University of Iowa (Iowa city, IA).

For growth curve assays, 50,000 cells were plated in triplicate and were treated with doxycycline or left untreated. The number of cells was counted on a daily basis for a week of treatment. The following drugs were used in cultured cells at the indicated concentrations: nocodazole (Sigma; 3.5 µM); taxol (Sigma; 1 µM), reversine (Sigma; 1 µM), ZM447439 (ZM1, 2 µM, Tocris Biosciences). Immortalization was achieved by retroviral infection with a plasmid enconding the first 121 aminoacids of the SV40 large T-antigen (T121) following hygromycin selection. To induce Aurora B expression, doxycycline was added at a final concentration of 1 µg/µl (D-9891, Sigma-Aldrich). Immortal murine MEFs were treated with doxycycline for a minimum of 24 hours in order to induce Aurora B expression. Wild-type or Aurora B inducible MEFs (untreated or doxycycline treated) were subjected to karyotyping as described in the previous section.

2.2. Induced pluripotent stem cell generation

Induced pluripotent cells (iPCs) were generated from i4F MEFs (Abad et al., 2013). In order to induce pluripotent cells the i4F MEFs were treated at passage 2 with iPCs medium consisting on: 15% of Knockout™ SR Serum Replacement for ES Cells, (Invitrogene, 10828-028; Lot. 719720), 1% non-esential aminoacids, 1% penicillin/streptomycin, 0.2% of 2-mercaptoethanol, and 0.1% LIF (10^3 u/ml final concentration). Doxycycline (1 µg/µl) was added to the iPCs medium for 15 days to induce the four Yamanaka's factors. Medium was changed every 1.5 days. Colonies were picked at day 15 and cultured in 96 well-feeders plate and were daily observed and subsequently passed.

2.3. Virus production and cell transduction

Lentiviruses and retroviruses were produced and concentrated as previously described (Tiscornia et al. 2006). Briefly, low passage 293T cells were transfected with the lentiviral vector of interest and the three packaging vectors expressing gag, pol and rev proteins necessary for virion production as well the envelope protein vsvg. 48 hours post transfection supernatants containing viruses were collected and concentrated by centrifugation at 19400 rpm for 2 hours at 20ºC. Viral pellets were resuspended in 1x HBSS. This viral preparation is of *in vitro* grade quality. For retroviruses production, 293T cells were transfected with the vector of interest and the packaging vector PCL-Eco. Supernatants were collected 48 hours post transfection. Packaging vectors are a gift from Dr. Verma´s lab. Transduction of primary MEFs were performed by adding 10 mL of concentrated virus (stock 10^9/mLl viral particles) per million of cells (MOI 10) for 12 hours or by using directly the viral supernatants collected from 293T cells repeating the virus addition three times for 6 hours.

2.4. Cell transfection

Plasmid transfection in MEFs was performed using Lipofectamine 2000 (Invitrogen) except when using OSKM MEFs transfection which was carried out using the Neon technology (Life Technologies; Invitrogen) using standard protocols.

3. Microscopy techniques

3.1. Live cell imaging

For time-lapse imaging experiments, synchronous, histone H2B-GFP-expressing inmortal MEFs were recorded using a computer- assisted microscope (images were adquired every 10-minute frames during 48 hours). Cells were prepared and after being plated on eight-well glass-bottom dishes (Ibidi). Video started after 18 hours upon doxycycline addition.Drugs were just added 2 hours before the video start. Time lapse acquisition was performed with a DeltaVision RT imaging system (Applied Precision; IX70/71;

Olympus) equipped with a charge-coupled device camera (CoolSNAP HQ; Roper Scientific) and a plan Apochromatic 40×/1.42 N.A. objective lens, and maintained at 37°C in a humidified CO_2 chamber. Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov) in order to determine the duration of mitosis (DOM).

3.2. Inmunofluorescence

The protocol for inmunofluorescence in cultured cells was adapted from (Perera et al., 2007). Adherent cells grown in coverslips were fixed for 7 min in freshly prepared 2.0% formaldehyde/PHEM at 37C (preheated). Cells were rinsed three times for 5 min with PBS containing 0.05% Tween-20 (PBST) and permeabilized in preheated 0.5% Triton X-100 for 10 min at 37C. Then cells were subsequently blocked for 45 min-1hour at RT in BSA1% PBS 1% and incubated overnight at 4C in a humid chamber with primary antibodies (shown in Table 4). The following day, after four 5-min rinses in PBST, cells were incubated for 45 min at room temperature in the dark, in secondary donkey anti–rabbit and anti-mouse antibodies at 1:250 (Jackson ImmunoResearch Laboratories), rinsed with PBST and stained with DAPI. Matching secondary antibodies with different Alexa dies (488, 594, 647) (Table 3). and DAPI for nuclei visualization were from Molecular Probes (4,6 diaminophenylindole, Prolong Gold antifade; Invitrogen). Image acquisition was performed using either a Leica D3000 microscope or confocal ultraspectral microscope Leica TCS-SP5-AOBS-UV.

Table 3. Secondary antibodies used in IF experiments.

Antibody	Type	IF	Source/Clone
Alexa Fluor-488	Donkey/goat anti-rabbit IgG (H+L)	1:1000	Invitrogen
Alexa-Fluor- 488	Donkey/goat anti-mouse IgG (H-L)	1:1000	Invitrogen
Alexa-Fluor- 594	Donkey/goat anti-mouse IgG (H-L)	1:1000	Invitrogen
Alexa-Fluor-594	Donkey/goat anti-human IgG (H-L)	1:1000	Invitrogen

4. Flow cytometry

For DNA content analysis, cells were fixed in 70% ethanol overnight at -20C. The following day cells were stained with propidium iodide (20 mg/mL; Sigma) in presence of RNAseA (0.2 mg/mL; Qiagen) for 30minutes at 4C and then analyzed by flow cytometry (Becton- DickinsonFranklin Lakes, NJ, USA). In order to determine S phase entry in splenocytes upon cell cycle stimulation entry with ConA and LPS, Edu analysis was performed. Cells were pulsed with EdU (10 μM, Sigma) for 30 minutes and then fixed in 70%

ethanol overnigth at -20ºC. The following day, DAPI (2 µg/ml, Sigma) was added and cells were analyzed by flow cytometry (Becton-Dickinson). Flow cytometric analysis was performed with a FACS-Canto flow cytometer or a LSRII flow cytometer (BD Biosciences) and FlowJo Version 8.8.7 software was used to analyze cell populations (TreeStar).

5. Biochemical procedures

5.1. RNA extraction and Real-Time PCR

To quantify expression of transcripts, total RNA from cells and tissues was isolated using Trizol (Invitrogen). Expression of *Aurkb* and *p21^{Cip1}* was quantified by real-time quantitative amplification with the SuperScript® III Platinum assay kit, according to the manufacturer's instructions, in a BioRad iCycler Real-Time PCR apparatus. The following primers were used: *Aurkb* FW: 5'-ATGGCTCAGAAGGAGAACGC-3', *Aurkb* RV: 5'-CCAGTTCCCACCCCTTCT-3'; *p21^{Cip1}* F 5'-CTAGGGGAATTGGAGTCAGGC-3', *p21^{Cip1}* RV: 5'-AACAGGTCGGACATCACCAG-3'. Amplification of α-GAPDH was used for normalization using the following oligonucleotides: *GAPDH* FW: 5'-GCCACCCAGAAGACTGTGGATGGC-3', *GADPH* RV: 5'-CATGATGGCCATGAGGTCCACCAC-3'. Data analysis was performed using the iQ5 v.2.0 software (BioRad).

5.2. Protein extraction and analysis

For immunodetection in protein lysates, cells and tissues were washed twice with ice-cold PBS and lysed in RIPA lysis buffer (37 mM NaCl, 0.5% NP-40, 0.1% SDS, 1% TX-100, 20 mM Tris-HCl pH 7.4, 2 mM EDTA, 10% glycerol 1 mM PMSF) or Laemmli buffer supplemented with protease and phosphatase inhibitory cocktails (Sigma). In the case of Ripa buffer, after 30 minutes on ice, samples were cleared by centrifugation, whereas Laemmli samples were directly denatured for 10 min at 95 degrees. 50 µg of total protein were separated on SDS-PAGE, transferred to nitrocellulose membranes (BioRad), and probed using specific primary antibodies that are noted as WB in Table 4. Secondary antibodies were coupled to HRP (DAKO) for immunodetection.

Table 4. Primary antibodies used in different assays.

Antibody	Host Species/Clonality	Application	Dilution	Source/Clone
ACA	Human (P)	IF	1:500	Antibodies Inc.
α-Actin	Mouse (M)	IF, WB	1:2000	Sigma / DM1A
Aurora B	Rabbit (P)	IF,IHQ,IP,WB	1:200	Abcam
Bax	Rabbit (P)	WB	1:200	Millipore
β-tubulin	Mouse (M)	WB	1:2000	Sigma/AC-40
Bromo-deoxyuridine	Mouse monoclonal	IHQ	1:50	GE Healthcare/BU-1
BubR1	Sheep	IF	1:100	S.Taylor (gift)
Caspase 3 active	Rabbit polyclonal	IHQ	1:200	RYD systems
CD3e (M-20)	Goat polyclonal	IHQ	1:500	Santa Cruz Biotec.
Cdk1	Mouse monoclonal	IP	1:200	Santa Cruz Biotec.
Cyclin B1	Rabbit polyclonal	WB	1:500	Santa Cruz Biotec.
Incenp	Rabbit polyclonal	WB	1:1000	Cell Signaling
Ki67	Rat monoclonal	IHQ	1:100	GE Healthcare
Mad2	Rabbit polyclonal	IF	1:200	K.Wassmann (gift)
Pax5 (C-20)	Goat polyclonal	IHQ	1:500	Santa Cruz Biotec.
Phospho-CENPA(ser 7)	Rabbit polyclonal	WB, IF	1:200	Rabbit polyclonal
Phospho-Histone H3 (Ser10)	Rabbit polyclonal	WB, IHQ	1:500/1:2000	Upstate Biotec.
γ-H2A.X (Ser139)	Mouse monoclonal	WB, IHQ	1:1000	Millipore / JBW30
Phospho-Th232-Aurora B	Rabbit polyclonal	WB, IF	1:200	Rockland
Phospho-p53 (Ser15)	Rabbit polyclonal	WB,IHQ	1:500	Cell Signaling
$P21^{Cip}1$	Mouse monoclonal	WB;IHQ	1:1000	Santa Cruz Biotec.
P53	Mouse monoclonal	WB	1:1000	Cell signaling
Plk1	Mouse monoclonal	IP	1:200	Abcam
PUMA	Rabbit polyclonal	WB	1:500	Abcam
Survivin	Rabbit polyclonal	WB	1:500	Novus Biologicals
Vinculin	Mouse	WB	1:5000	Sigma

P: polyclonal, M: monoclonal; Immunohistochemistry (IHQ), Immunofluorescence (IF), Immunorecipitation (IP) and Western blot (WB)

6. DNA damage assays

To induce DNA damage, *Aurkb*$^{+/+}$*Rosa26*$^{M2rtTA/M2rtTA}$ and *Aurkb*$^{+/tet}$ *Rosa26*$^{M2rtTA/M2rtTA}$ immortal murine MEFs were treated with doxycycline for 24 hours in order to induce Aurora B. 1 μM Adryamicin was then added for two hours to induce p53 signaling. Samples were taken 6 and 10 hours post-Adryamicin treatment. For inducing DNA damage *in vivo*, *Aurkb*$^{+/+}$; *Rosa26*$^{M2rtTA/M2rtTA}$ and *Aurkb*$^{+/tet}$ *Rosa26*$^{M2rtTA/M2rtTA}$ mice were treated for fifteen days with doxycycline and unanaesthestized animals were restrained in well-ventilated perspex boxes and whole body exposed to a single dose of 8 Gy gamma radiation. Animals were sacrified 24 hours after the irradiation procedure. Labeling retaining experiments (BrdU) were based on previously reported procedures (Braun et al., 2003).

7. Physiological and metabolic assays

7.1. Dual Energy X-ray Absortiometry (DEXA)

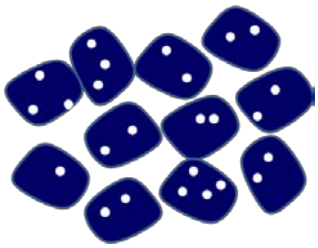
The percentage of body lean mass was determined from whole-body samples (excluding cranial region) using a Lunar Piximus densitometer (GE medical systems) with a high degree of accuracy. DEXA was performed just before the metabolic measures (OXYLET) were taken.

7.2. Metabolic cages

The Oxylet (Harvard Apparatus) allowed us to measure three different parameters related to metabolism: 1. Indirect calorimetry; 2. Food/drink intake; 3. Locomotive activity. 1. Indirect calorimetry is a method used to measure the energy consumption of animals. It is based on the calculation of the energy produced by the cellular metabolism through the products of respiratory exchange. The apparatus monitors respiratory metabolism: O_2 consumption and CO_2 production and from these parameters we calculated the respiratory quotient (RQ), which is calculated by dividing Vc_{02} production/V_{O2} consumption. These values were used to calculate the mice energy expenditure (EE). The equation used for this calculation is the semi-empirical Weir Equation. EE is given in kcal/day kg·3/4 (Weir, 1949). 2. Food and drink consumption was measured the whole day. These data were always relating to the total body weight of the mouse or the total body lean mass. 3. Locomotive activity was measured by analyzing two parameters: mean spontaneous activity of the mouse along the cage and number of rearings.

8 Statistical and imaging analysis

Statistical analysis was performed using Student's t-test, Fisher's exact test, or log-rank tests (GraphPad Prism 5). EE was analysed by using ANCOVA and the variable weight as co-variant. All data are shown as mean ± SEM. Probabilities of $p<0.05$ or $alpha<0.05$ (Fisher's exact test) were considered significant. Images were quantified using ImageJ. In most figures, *, $p<0.05$; **, $p<0.01$; ***;$p< 0.001$.



Results

Results

Aurora B function has been extensively studied in yeasts, nematodes, insects and cold-blood vertebrates but very limited information concerning the Aurora B role in mammals and its implication in tumourigenesis is available. To study the *in vivo* function of Aurora B in mammals, two different mouse models were generated in our laboratory originally by Fernández-Miranda G.: a conditional knock-out model and a tetracycline inducible model (Figures 11 and 20).

1. Conditional Aurora B depletion model

1.1. Generation of conditional Aurora B depleted mice

The conditional knock-out model was generated by using a targeting vector in which Aurora B exons 2-6 were flanked with loxP sequences and a frt-neo^r (neomycin-resistant gene)-frtv cassette for selection purposes (Figure 11). At both ends of the construct two homology arms were cloned to facilitate HR in ES cells. Clones carrying the recombinant allele *Aurkb*(loxfrt) (Fig 11b) were selected and the corresponding ES cells were microinjected in wild-type blastocysts to generate *Aurkb*(+/loxfrt) mice. The neo^r cassette was first removed by crossing with transgenic mice expressing the Flp recombinase resulting in the conditional *Aurkb*(lox) allele. Deletion of exons was then achieved by additional crosses with CMV-Cre transgenic mice to generate *Aurkb*(-). Elimination of exons in *Aurkb*(-) allele ensures total inactivation of Aurora B since the kinase domain of Aurora B is almost completely removed. By using the conditional *knock out (cKO)* model for Aurora B (Fernández-Miranda et al., 2011), it was observed that the genetic elimination of Aurora B *in vitro* induces problems during prometaphase avoiding the correct segregation of chromosomes during mitosis, leading to abnormal interphasic and polyploid cells. Moreover, complete ablation of Aurora B is embryonic lethal whereas heterozygous mice are fertile but have shorter lifespan (Fernández-Miranda et al., 2011). Here we use this depletion model to study the *in vivo* effect of the complete depletion of Aurora B in adult mammals.

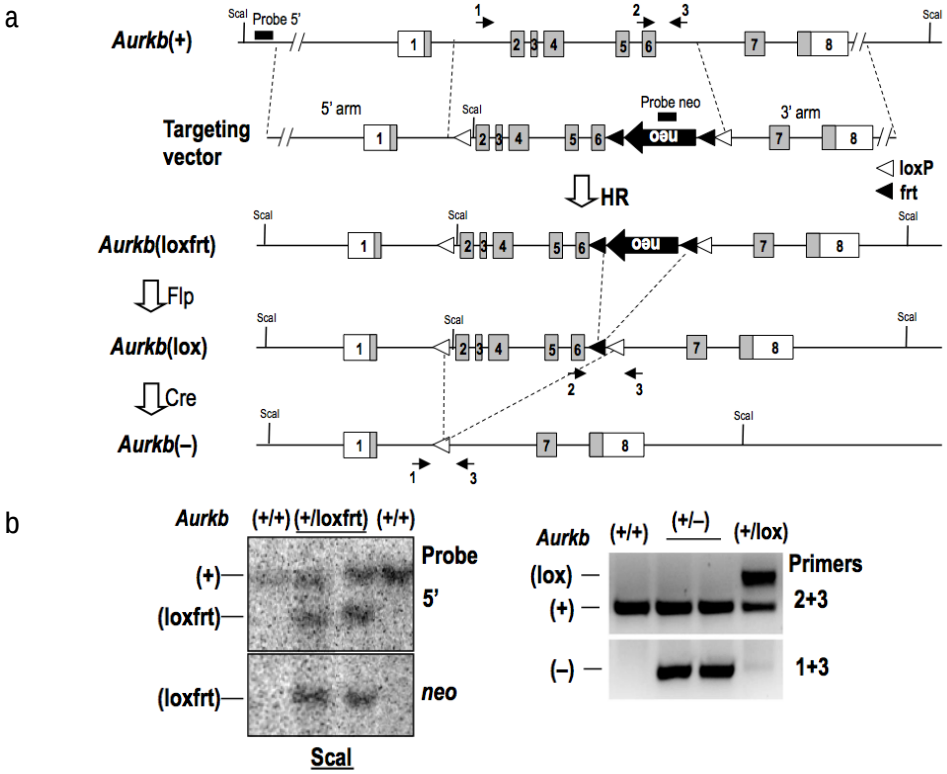


Figure 11. Aurora B conditional knockout alleles. (a) Schematic representation of *Aurkb* null knock-out alleles.The mouse *Aurkb* locus encoding Aurora B contains 8 exons (boxes) containing non-coding (open boxes) or protein coding (filled boxes) sequences. Wild-type (white triangles) loxP sites and frt (black triangles) sites are used to flank Aurora B exons or the neo resitance in the targeting vector. The neo cassette (black) contains promoter and poly-A sequences and it is used for selection of clones after homologous recombination (HR) in ES cells. The neo cassette is eliminated *in vivo* by crossing *Aurkb*(+/loxfrt) mice with transgenic mice expressing the Flp recombinase. Further excision of exons 2-6 is mediated by expression of the Cre recombinase resulting in the Aurkb(-) null allele. The ScaI restriction sites are also shown, 5' and neo probes and primers 1,2,3 used for genotyping. (b) Southern blot analysis of recombinant ES cells showing two *Aurkb*(+/loxfrt) clones that underwent HR. DNA was digested with ScaI and hybridized with the probe 5' or neo specific sequences (Fernández-Miranda et al., 2011).

1.2. Decreased survival of Aurora B depleted mice

The effects produced by the ablation of Aurora B in adult mammals have not been studied so far. To address this fundamental question, we took advantage of the conditional mouse model for the depletion of *Aurkb* (Fernández-Miranda et al., 2011) that allowed us to study the *in vivo* defects caused by Aurora B depletion in the whole animal.

We first fed wild type and transgenic mice with tamoxifen for a month in order to ensure that we were able to deplete Aurora B *in vivo*. As shown in Figure 12a, a significant reduction in the expression of Aurora B protein in different tissues was observed in *Aurkb*$^{lox/lox}$*; UbCre*T mice. Then, in order to analyse the consequences of Aurora B ablation in an adult organism *Aurkb*$^{++;}$*UbCre*T and *Aurkb*^{loxlox}*UbCre*T mice were fed with tamoxifen since weaning for 80 weeks. This significant reduction of Aurora B expression compromised the survival of adult mice since by one year of age, almost 40% of the Aurora B-deficient mice had died or had to be sacrificed whereas all control mice survived (Figure 12b).

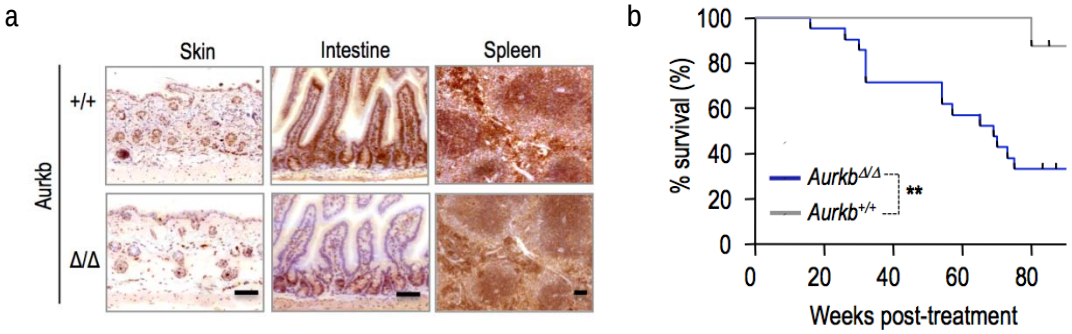


Figure 12. Aurora B depletion in adult mice induces decreased survival as a consequence of diminished proliferation and increased apoptosis. (a) Aurora B inmunohistochemical detection is shown for the skin, intestine and spleen of wild-type (+/+) and Aurora B null (Δ/Δ) mice. Aurora B expression is significantly diminished upon tamoxifen treatment in *Aurkb*$^{lox/lox}$UbCreT mice. (b) Aurora B deletion results in lethality.Graph shows the survival curve from 1 to 20 months of tamoxifen treatment. Survival curves of *Aurkb*$^{+/+}$UbCreT mice (n=10) and *Aurkb*$^{lox/lox}$UbCreT (n=19) mice were compared using a log-rank (Mantel-Cox) test. ** p< 0.01. Scale bars 50 µm.

1.2.1. Impaired proliferation and increased apoptosis in Aurora B depleted mice

When analyzing in more detail several proliferative tissues such as spleen or intestine, we observed that Aurora B depletion produced a significant reduction in the proliferation marker Ki67. A detailed analysis of these proliferative tissues allowed us also to visualize among the *Aurkb*$^{Δ/Δ}$ tissues a significant increase in the levels of the apoptotic marker Active-Caspase3 (AC3) (Figure 13). These data indicated us that Aurora B depletion was impairing proliferation and increasing apoptosis.

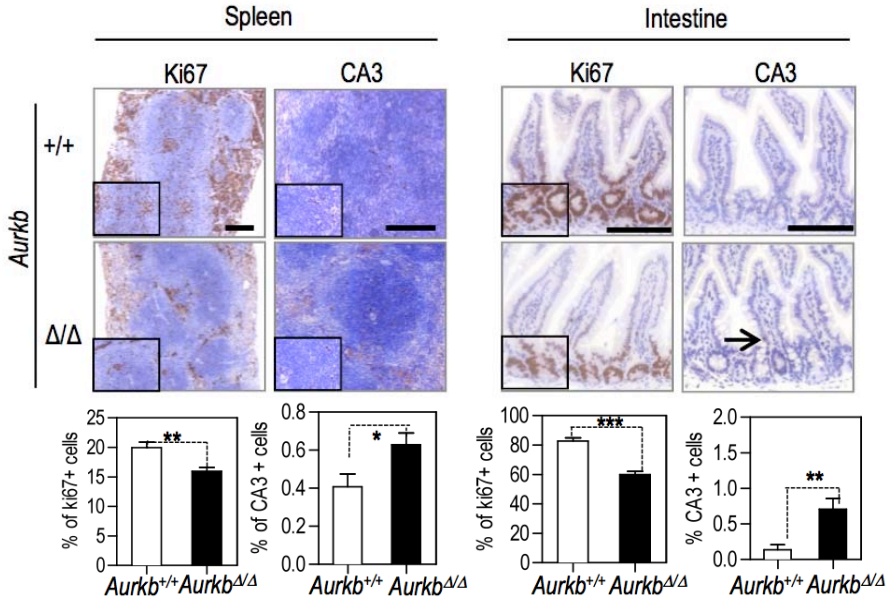


Figure 13. Aurora B depletion impairs proliferation and induces apoptosis. Representative pictures of inmunohistochemical detection for Ki67 and Active caspase-3 are shown for intestine and spleen of wild-type (+/+) and Aurora B null (Δ/Δ) mice. Ki67 expression is significantely decreased in both tissues upon tamoxifen treatment in *Aurkb*$^{lox/lox}$UbCreT mice, versus wild-type mice (19.98 ± 0.92 v. 15.97 ± 0.63; spleen and 82.68 ± 2.18 v. 59.98 ± 2.19; intestine).CA3 expression is increased in *Aurkb*$^{lox/lox}$UbCreT mice, versus wild-type mice (0.41 ± 0.07 v. 0.63 ± 0.06; spleen) (0.14 ± 0.07 v. 0.70 ± 0.15; intestine). Bar graphs indicate the quantification of the number of positive cells for Ki67 and Caspase3 (n=3 mice per genotype). Scale bars 100 µm *, p< 0.05;** p< 0.01; *** p< 0.001.

1.2.2. Aurora B depleted mice are defective in hematological parameters

Defects in bone marrow, such as medullar fibrosis and loss of cellularity were characteristic of null mice for Aurora B. When analyzing peripheral blood, red and white blood cells were significantly reduced in the Aurora B depleted mice after 60 weeks of tamoxifen treatment (Figure 14a), which is in accordance to the lack of cellularity in the medulla. Moreover, We observed lack of proliferation and increased apoptosis in the bone marrow of Aurkb$^{\Delta/\Delta}$ mice (Figure 14b) No differences in bone density were observed by densitometry analysis (data not shown), probably as a consequence of the increase fibrosity of the bone marrow.

a

Table 1. Aurora B-deficient mice are defective in several hematological parameters

	Aurkb$^{+/+}$ (n=7)		*Aurkb*$^{\Delta/\Delta}$ (N=7)		
	Mean	**SEM**	**Mean**	**SEM**	**p**
WBC (x 10^9/L)	11.42	1.08	4.37	0.68	***
LYM (x 10^9/L)	7.94	0.80	2.68	0.37	***
MID (x 10^9/L)	0.45	0.17	0.16	0.08	0.16
GRA (x 10^9/L)	3.03	0.55	1.54	0.32	*
RBC (x 10^{12}/L)	9.64	0.91	8.33	0.22	0.18
HGB (g/dL)	15.40	1.70	13.10	0.40	0.21
HCT (%)	48.15	5.01	39.81	1.19	0.22
MCV (fl)	49.86	0.63	49.86	0.77	1.00
MCH (pg)	15.84	0.24	15.61	0.25	0.72
MCHC (g/dL)	31.90	0.52	31.37	0.32	0.62
RDWc(%)	19.03	1.21	18.34	0.16	0.44
PLT(x 10^9/L)	428.71	82.31	623	118.89	0.13
PCT(%)	0.29	0.04	0.47	0.08	0.06
MPV(fl)	7.03	0.34	7.40	0.23	0.28
PDWc	31.66	1.28	33.43	0.84	0.32

NOTE: * p< 0.05; *** p< 0.001
Abbreviations: GRA, granulocites count; HCT, hematocrit; HGB, hemoglobin; LYM, lymphocyte count; MCH, mean corposcular hemoglobin; MCHC, mean corposcular hemoglobin concentration; MCV, mean corposcular volume; MID, monocytes/eosionophils count; MPV, mean platelet volume; PCT,platelet percentage; PDWc platelet distribution width; PLT, platelet count; RBC, red blood cell count, RDWc red cell distribution width; WBC,total white blood cell count.
(65-80 weeks old mice)

b

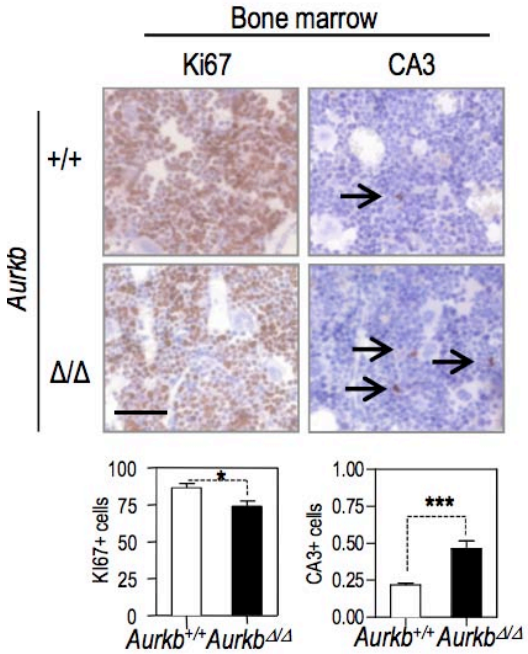


Figure 14. Conditional ablation of Aurora B in adult mice affects hematological parameters. (a) Hematological parameters in wild-type and Aurora B null mice showing reduced number of white blood cells in the Aurora B null mice (Aurkb$^{\Delta/\Delta}$). (b) Inmunohistochemical detection for Ki67 and Active Caspase-3 is shown in the bone marrow of wild-type (Aurkb$^{+/+}$) and null (Aurkb$^{\Delta/\Delta}$) mice showing decreased Ki67 expression and increased number of apoptotic cells upon tamoxifen treatment in Aurora B null mice. (86.44 ± 2.82 v. 74.07 ± 3.57; Ki67 staining) (0.21 ± 0.00 v. 0.47 ± 0.05; AC3 staining).Bar graphs indicate the quantification of the number of Ki67 and Caspase3 positive cells (n=3 mice per genotype). Scale bars 50µm.*, p< 0.05, *** p< 0.001.

1.3. Aurora B depletion leads to aneuploidy and increased p53 response

1.3.1. Lack of Aurora B leads to aneuploidy

The *Aurkb*$^{\Delta/\Delta}$ spleen red pulp was characterized by the presence of cells with an increased nuclear size (anisocariosis) (Figure 15a). This phenotype was also detected in other cell types such as kidney, pancreas and liver. The observed anisocariosis was an indication of division defects that may reflect an abnormal number of chromosomes caused by the depletion of Aurora B in the whole animal. We also analysed aneuploid levels among the ageing mice; peripheral blood lymphocytes were taken from control and Aurora B depleted mice and interphasic nuclei were subjected to fluorescence in situ hybridization (FISH) analysis using probes against chromosomes 8 and 11. The percentage of aneuploidy, calculated as the deviation from the mode, was not significantly different when analyzing *Aurkb*$^{+/+}$ and *Aurkb*$^{\Delta/\Delta}$ mice aged for 4 months (p=0.08). At 20 months of age, these numbers increased and the difference between both genotypes was statistically significant (3.6% of aneuploidy in *Aurkb*$^{+/+}$ and 9.7% of aneuploid cells in *Aurkb*$^{+/tet}$ mice (*,p= 0.0447). Age-dependent accumulation of aneuploidy in lymphocytes was observed in both genotypes although *Aurkb*$^{\Delta/\Delta}$ animals contained a higher percentage of aneuploidy (Figure 15b).

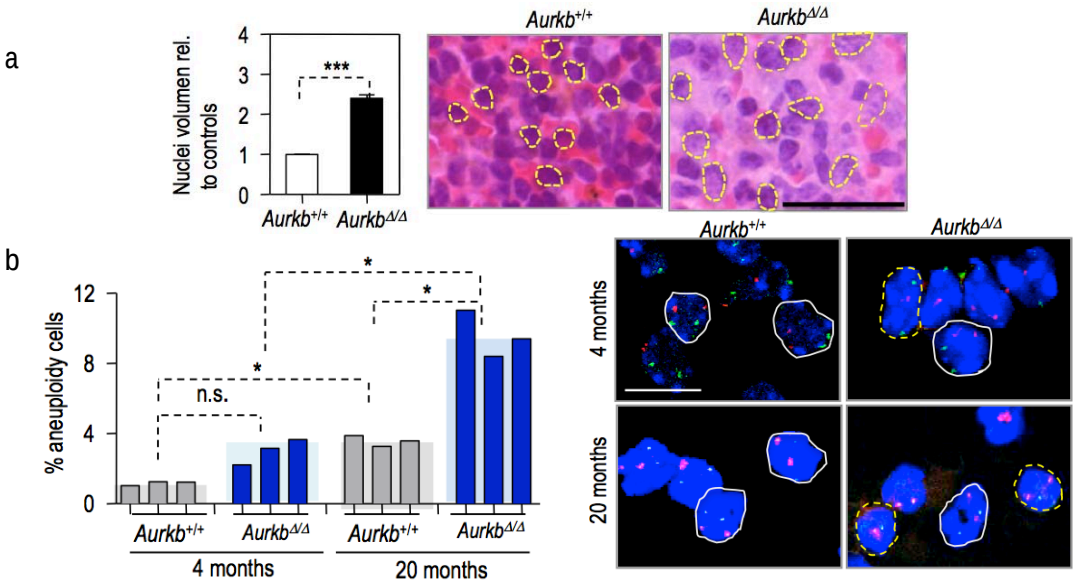


Figure 15. Aurora B depletion leads to aneuploidy. (a) Aneuploidy is a common feature in spleens of *Aurkb*$^{\Delta/\Delta}$ mice. Nuclear size was used to compare DNA content of Aurora B defective and control cells. Images show H&E spleen sections where nuclei have been highlighted. Scale bar, 50 µm. Bar graphs shows the quantification of the nuclear volumes of Δ/Δ samples (n= 150 nuclei per condition) normalized to control cases (2.40 ± 0.09 fold change versus wild-type mice). (b) *In vivo* aneuploidy study in *Aurkb*$^{+/+}$ and *Aurkb*$^{\Delta/\Delta}$ along tamoxifen treatment. *Aurkb*$^{\Delta/\Delta}$ mice contain high levels of aneuploid cells that are not eliminated from the cycling population. Aneuploidy was scored by counting the number of FISH signals per cell in lymphocytes from 4 and 20-month-old animals (At least n= 100 nuclei per genotype and time point). FISH analysis was performed using probes for chromosomes 8 (red) and 11 (green). Dot lines represent aneuploid cells. The percentage of aneuploidy was 1.14 % in *Aurkb*$^{+/+}$ and 2.69% in *Aurkb*$^{\Delta/\Delta}$ when the mice were 4 months old (p= 0.08) and 3.6% in Aurkb$^{+/+}$ and 9.7% in *Aurkb*$^{\Delta/\Delta}$ when the mice were aged 20 months (p= 0.0447). Each column represents one animal. Data are shown as the deviation from the mode. Representative FISH images are shown on the right panel. Scale bar,20 µm. *, p< 0.05; ***, p< 0.001.

1.3.2. Increased p53 signaling upon Aurora B depletion

Since p53 and its main target p21^{Cip1} are induced in vitro upon Aurora B depletion (Trakala et al., 2013) we decided to test whether depletion of Aurora B *in vivo* was also accompanied by p53 activation. As represented in Figure 16, both p53 and its effector p21^{Cip1} were upregulated in the spleen of *Aurkb*$^{\Delta/\Delta}$ mice compared to wild-type animals.

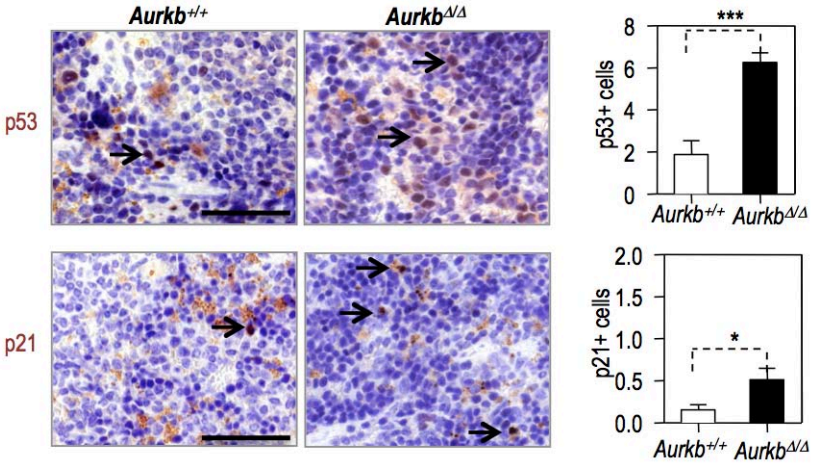


Figure 16. In vivo depletion of Aurora B induces p53 signaling. p53 and p21 upregulation by Aurora B. Images and IHC quantification from spleen samples collected from *Aurkb*$^{+/+}$ and *Aurkb*$^{\Delta/\Delta}$ mice are displayed. The percentage of positive cells was evaluated for p21 and p53. As shown, the percentage of p21and p53 positive cells was higher in *Aurkb*$^{\Delta/\Delta}$ mice (6.26 ± 0.45 v. 1.87 ± 0.67; p53 staining) (0.51 ± 0.14 v. 0.15 ± 0.06; p21 staining). Scale bar, 50 μm. .*, p< 0.05; ***, p< 0.001.

1.4. Premature ageing in Aurora B depleted mice

The main phenotype observed among the Aurora B-deficient mice was premature ageing (Figure 17a), 85% of *Aurkb*$^{\Delta/\Delta}$ mice presented this phenotype whereas it was not found in any of the wild-type animals (Figure 17a). The external phenotypes of *Aurkb*$^{\Delta/\Delta}$ mice were loss of hair, hair graying and kyphosis, hallmarks typically associated with an ageing phenotype (Figure 17a). Histopathological analysis of sick mice showed a pleiotropic phenotype preventing us from establishing a single common cause of death in all the cases. Several features found in Aurora B null mice such as thinner and disorganized epidermis, kidney inflammation and degeneration, extramedullary hematopoiesis, spleen an intestine atrophy, can be associated to death as a result of starvation, infection, dehydratation or multiorganic failure. Aurora B depleted mice also presented cataracts, adipose atrophy, medullar fibrosis and pancreas, liver and kidney anisocariosis (Figure 17b and 17c). The intestine showed decreased number of crypts and villi length. Reduced follicles in the spleen of *Aurkb*$^{\Delta/\Delta}$ could also be observed.

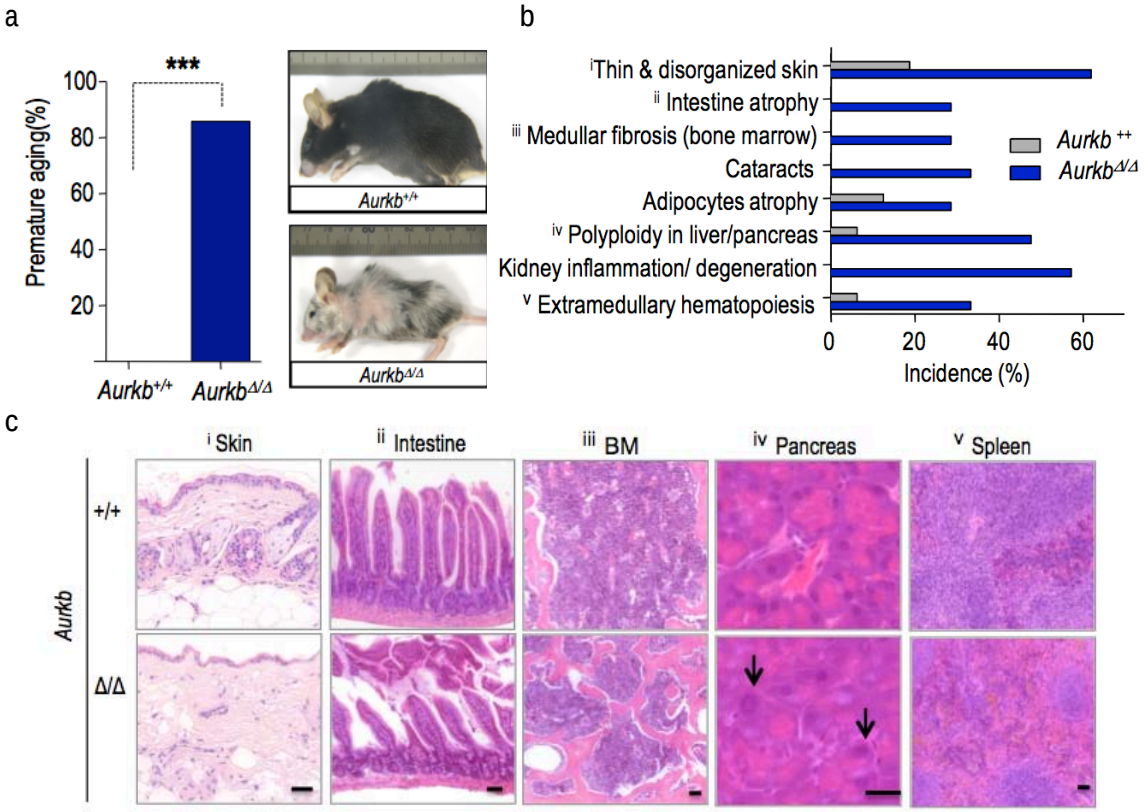


Figure 17. Aurora B depletion in adult mice induces premature ageing. (a) Premature ageing incidence found in the Aurora B-depleted mice is shown. *Aurkb*$^{\Delta/\Delta}$ ***, p=0.0001 vs. wild-type mice using Fisher's exact test. Right panel shows the external view of *Aurkb*$^{lox/lox}$UbCreT and *Aurkb*$^{+/+}$UbCreT mice treated with tamoxifen during 50 weeks. (b) Graph shows the percentage of incidence of the ageing pathologies found in the Aurora B-depleted mice (c) H&E staining of the indicated pathologies found in *Aurkb*$^{lox/lox}$UbCreT animals: i: skin atrophy; ii: abnrnomal intestine cripts; iii: medullar fibrosis; iv pancreas anisocariosis; v: extramedullar hematopoyesis. Scale bar, 50 µm.

1.5. Aurora B depletion prevents tissue regeneration

1.5.1 Diminished proliferative capacity of Aurora B depleted mice

To investigate the consequences of Aurora B ablation in tissue regeneration *in vivo*, we made use of the hair follicle cycle. *Aurkb*$^{+/+}$ and *Aurkb*$^{\Delta/\Delta}$ mice were subjected to skin hair removal on 2cm^3 patches of their back skin and were next treated with tamoxifen for 6 days. A week later, control mice were able to repopulate their back skin with new hair, this was not the case for *Aurkb*$^{\Delta/\Delta}$ mice (Figure 18a). Particularly, the epidermis of *Aurkb*$^{\Delta/\Delta}$ mice presented clear cell depletion and hair follicles were mainly in the catagen-telogen stage without reaching the panniculus carnosus muscle. Aurora B depletion was clearly visualized (Figure18a). When analyzing further the skin of the mutant mice we observed a significant increase in the number of mitotic cells in the follicle hair, being most of them metaphases; abnormal mitosis such as misaligned chromosomes could also be observed in the skin of *Aurkb*$^{\Delta/\Delta}$ mice. These data indicated us that probably Aurora B depletion is causing division defects thus preventing a correct cell division, resulting in tetraploidization.

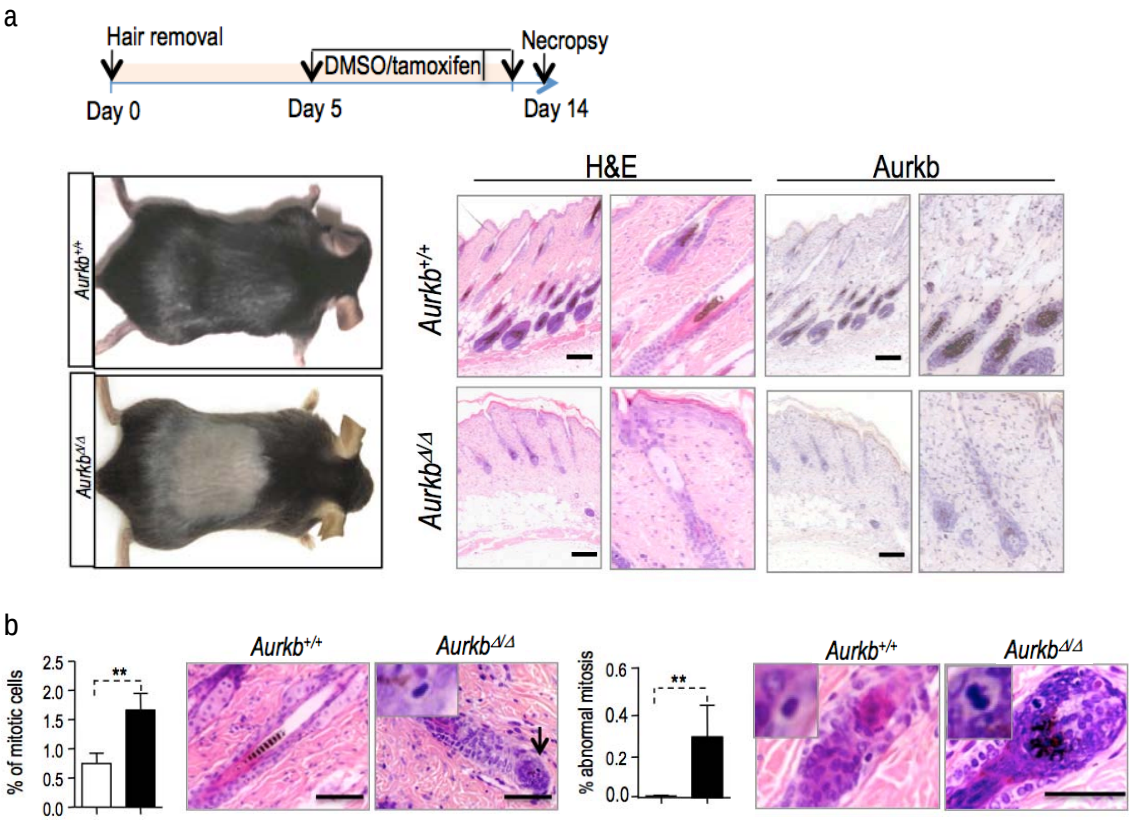


Figure 18. Lack of Aurora B affects hair regeneration. (a) Scheme of the protocol followed to test the regenerative capacity of Aurora B depleted hair follicles. The back skin of *Aurkb*$^{+/+}$ (n=3) and *Aurkb*$^{\Delta/\Delta}$ mice (n=3) was removed and topically treated with tamoxifen. Pictures show how 1 week later, hair recovery was observed in control mice whereas no hair repopulation was observed in *Aurkb*$^{\Delta/\Delta}$ mice. On the right panels, hematosilin and eosin staining (H&E) showing the arrest of hair follicle cycle in catagen-telogen stage and Aurkb IHCs showing the depletion of the protein in the skin of transgenic mice.(b) Hematosilin and eosin staining shows an increase in the number of mitosis in the skin of *Aurkb*$^{\Delta/\Delta}$ mice (0.76 ± 0.13 v. 1.66 ± 0.28). On the right panel, the percentage of abnormal mitosis (0.003 ± 0.003 v. 0.29 ± 0.15) and pictures showing these defects are shown. Scale bar, 200 µm in (a) and 50 µm in (b). **, p< 0.01.

1.5.2. Lack of stem cells regeneration and apoptosis upon Aurora B depletion

Moreover, a significant increase in the number of apoptotic cells could be observed in the skin of the Auora B depleted mice, which impinges on the regenerative capacity of the hair follicles (Figure 19a). Moreover, when analyzing the stem cell marker CD34, we found a decrease in the number of positive cells for this marker in the Aurora B-deficient mice (Figure 19b). These data indicated us that the lack of Aurora B was producing problems in mitosis that lead to an increase in the ratio of apoptotic cells and at the same time was affecting the regenerative capacity of stem cells to repopulate the tissue.

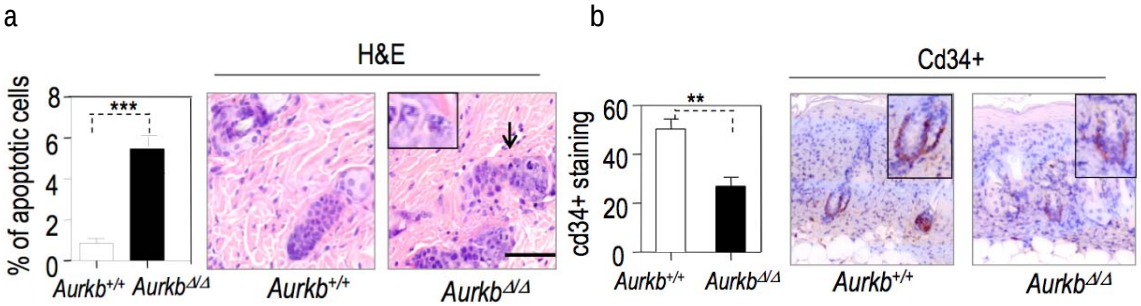


Figure 19. Aurora B depletion produces lack of stem cell regeneration and induces apoptosis. (a) Inmunohistochemistry analysis of the stem cell marker Cd34 is shown in wild-type and Aurora B-depleted mice (1.47 ± 0.05 in wild-type mice v. 5.76 ± 0.67 positive cells in *Aurkb*$^{\Delta/\Delta}$).(b) Percentage of apoptotic cells in the skin of of *Aurkb*$^{+/+}$ (n=3) and *Aurkb*$^{\Delta/\Delta}$ mice (n=3) is represented, (51.26 ± 0.52 in wild-type v. 23.87 ± 0.37 *Aurkb*$^{\Delta/\Delta}$). Images are shown on the right. Scale bar 100 µm.

2. A model for Aurora B overexpression

2.1 Generation of an endogenous Aurora B inducible model

Due to the fact that Aurora B is generally over-expressed in a variety of human tumours (Chieffi et al., 2006) and that its deregulation is related to aneuploidy and therefore to cancer, our goal is to know whether Aurora B overexpression can lead to tumour formation through aneuploidy induction. To achieve this first objective, a murine allele in which the expression of the endogenous Aurora B gene, *Aurkb*, can be induced *in vitro* and *in vivo* was generated in collaboration with Proffesor Earnshaw's laboratory at the Wellcome Trust Center for Cell Biology in Edinburgh.

To generate a model with inducible expression of Aurora B, the promoter region of the corresponding murine gene was genetically modified (*Aurkb*; Figure 20a). This model is based on the hijacking promoter strategy previously used in chicken DT40 cells in which the endogenous *Aurkb* promoter (2 kb upstream of the translation initiation codon ATG of Aurkb gene) is substituted with a minimal cytomegalovirus (CMV) promoter-linked with seven in tandem repeats of tetO sequences. These sequences are flanked by loxP sites and a puromycin cassette that was used for positive selection of the clones (Samejima et al., 2008). This construction leads to a tetO-CMV promoter (Tet-P) that is only active in the presence of the binding of the regulatory proteins (rTTA) to the tetO sequences.

After homologous recombination in ES cells, recombinant clones (*Aurkb* lox-tet) that were puromycin resistant, were selected by Southern blot analysis using the 5' and neo probes as indicated in Figure 20. These clones were aggregated into developing blastocysts togenerate *Aurkb*$^{+/loxtet}$ mice (chimeras were generated through standard blastocyst microinjection). The puromycin-resistant cassette was then removed by crossing with transgenic mice expressing the Cre recombinase resulting in the *Aurkb*$^{+/tet}$ mice (Figure 20a). We were not able to obtain homozygous *Aurkb*$^{loxtet/loxtet}$ or *Aurkb*$^{tet/tet}$ mutants from crosses between heterozygous mice, in agreement with a lethal phenotype caused by the lackof Aurora B in embryos.

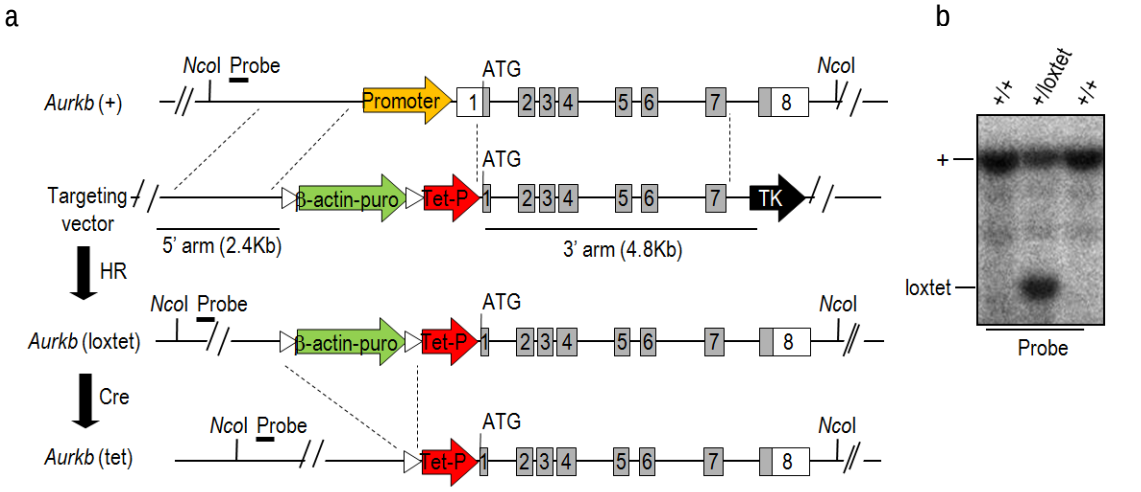


Figure 20. Inducible model for Aurora B in which *Aurkb* promoter has been replaced by a tetracycline-responsive minimal promoter (a) Schematic representation of the alleles used in this study for the inducible overexpression of Aurora B (see Matherial and Methods for details). In the *Aurkb*loxtet allele, the Aurora B endogenous promoter was replaced by a tet-P, a minimal CMV promoter that contains seven *in tandem* repeats of tetO sequences recognized by the transactivator regulatory proteins rtTA. The activity of Cre recombinase resulted in the removal of the puromycin-resistance cassette and the generation of the *Aurkb*tet allele. (b) Southern blot analysis of ES cell clones after homologous recombination indicates the presence of the recombinant *Aurkb*loxtet allele in one of the clones. The location of the probe is indicated in (a).

Finally, to generate an inducible system, the Aurora B inducible mice were crossed with mice expressing the Rosa26 (rtTA) allele (Beard et al., 2006), which expresses a tetracycline-inducible M2rtTA transactivator driven from the endogenous ubiquitous Rosa26 promoter.

Addition of tetracycline modifies the configuration and binding of rtTA to the tetO sequences so that rtTA binds to and activates expression of the tetP (Figure 21). For *Aurkb* induction, mutant and wild-type mice (as controls) were fed with doxycycline-supplemented food so that *Aurkb* is overexpressed in the whole animal.

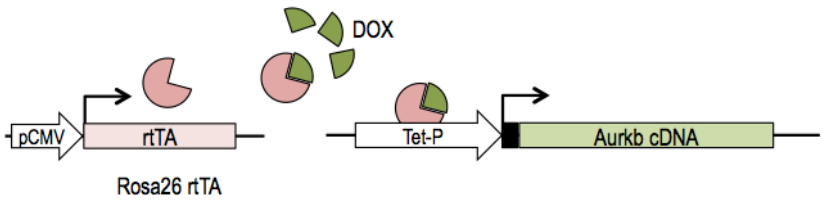


Figure 21. Inducible expression of Aurora B (TetON system). The *Aurkb*(tet) allele contains a minimal tetO-CMV promoter (Tet-P) instead of Aurora B endogenous promoter. The tet-P sequences are recognized by transactivator regulatory proteins (rtTA). The Rosa26(rtTA) allele generated in (Beard et al., 2006) expresses a tetracycline-inducible M2rtTA transactivator driven from the endogenous ubiquitous Rosa26 promoter. Addition of tetracycline modifies the configuration and binding of rtTA to the tetO sequences so that rtTA binds to and activates expression of tet-P in the presence of tetracycline (Tet-ON system).

2.2. Aurora B overexpression results in mitotic defects and aneuploidy

2.2.1. *In vitro* characterization of Aurora B overexpressing MEFs

Aurkb$^{+/tet}$; *Rosa26*$^{M2rtTA/M2rtTA}$ and *Aurkb*$^{+/loxtet}$; *Rosa26*$^{M2rtTA/M2rtTA}$ mouse embryonic fibroblasts (MEFs) were obtained and cultured in the absence or presence of doxycycline. Treatment with doxycycline induced a significant increase in Aurora B mRNA and protein levels (Figure 22 a and b). Aurora B was always induced to a higher level in *Aurkb*$^{+/tet}$ than in *Aurkb*$^{+/loxtet}$ MEFs, and we therefore selected *Aurkb*$^{+/tet}$ clones for further assays. The doxycycline treatment did not alter the localization of Aurora B (Figure 22c) indicating that Aurora B overexpressed protein was functional and located properly.

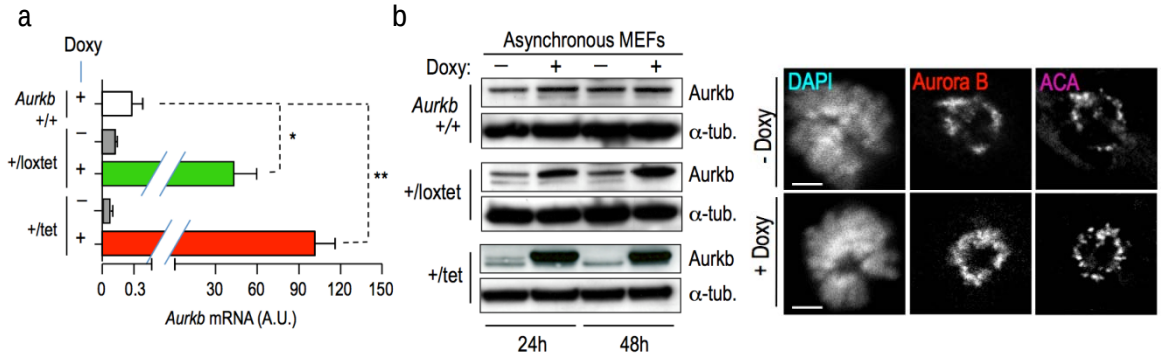


Figure 22. Aurora B overexpression in MEFs. (a) Quantitative RT-PCR data from *Aurkb*$^{+/+}$, A*urkb*$^{+/loxtet}$ and *Aurkb*$^{+/tet}$ cells carrying the *Rosa26(*M2rtTA/M2rtTA) allele in the absence or presence of doxycycline (doxy) for 48h. GADPH was used as control of expression. (b) Immunodetection of Aurora B in *Aurkb*$^{+/loxtet;}$ *Rosa26*$^{M2rtTA/M2rtTA}$ and *Aurkb*$^{+/tet;}$ *Rosa26*$^{M2rtTA/M2rtTA}$ primary mouse embryonic fibroblasts (MEFs) stimulated with doxycycline. On the right, detection of Aurora B (red) by immunofluorescence in the absence or presence of doxycycline. Anti-centromere antigen (ACA; purple) was used as a control. DAPI (blue) was used to stain DNA. Scale bars, 10 μm.

Since Aurora B forms part of the CPC complex we decide to analyse the levels of its partners upon overexpression of Aurora B. No changes in the protein levels of its CPC partners INCENP or Survivin were observed (Figure 23).

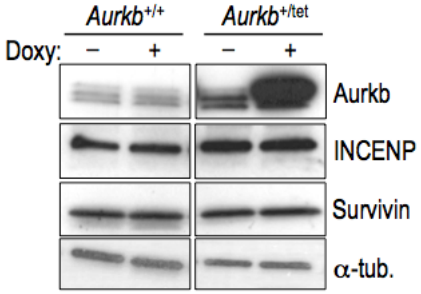


Figure 23. CPC partner protein levels upon Aurora B overexpression. Protein levels of the CPC components in Aurora B-overexpressing and control MEFs. α-tubulin was used as a loading control.

Induction of Aurora B was also evident in non-cycling lymphocytes (Figure 24) and serum-starved MEFs (Figure 24) treated with doxycycline, indicating that Aurora B gene induction in this system is independent of cell cycle regulation.

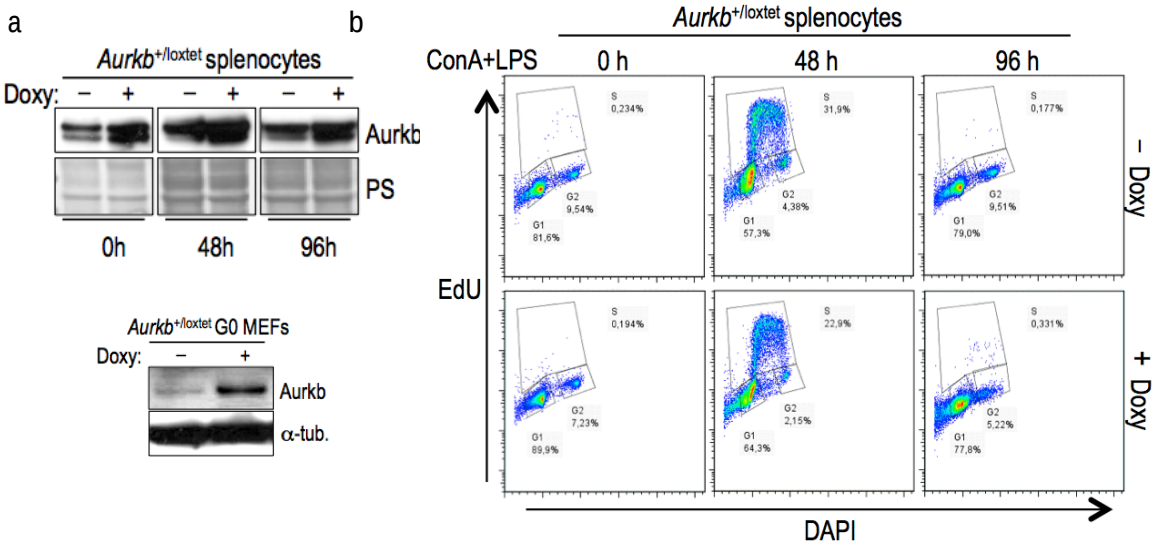


Figure 24. Cell cycle independent-overexpression of Aurora B. (a) Aurora B expression is significantly induced in *Aurkb*$^{+/loxtet}$ resting (0h) or concanavalin A + lipopolysaccharide (ConA+LPS)-induced (48 and 96h) splenocytes. PS, Ponceau S. At the bottom, Aurora B levels in serum-starved *Aurkb*$^{+/loxtet}$; *Rosa26*$^{M2rtTA/M2rtTA}$ MEFs treated or not with doxycycline for 48h. (b) Edu staining in stimulated Aurora B overexpresing lymphocytes. EdU incorporation and DNA content profiles in *Aurkb*$^{+/loxtet}$ splenocytes before or after treatment with concanavalin A + lipopolysaccharide (ConA+LPS).

2.2.2. Impaired proliferation of Aurora B overexpressing MEFs

Treatment of *Aurkb*$^{+/tet}$; *Rosa26*$^{M2rtTA/M2rtTA}$ MEFs with doxycycline for 5 days resulted in impaired proliferation when compared to untreated *Aurkb*$^{+/tet}$ clones or control *Aurkb*$^{+/+}$ fibroblasts (Figure 25). The proliferation of untreated *Aurkb*$^{+/tet}$ MEFs was probably reduced compared to wild-type cells as a consequence of the presence of a single functional Aurkb allele in these mutant cells.

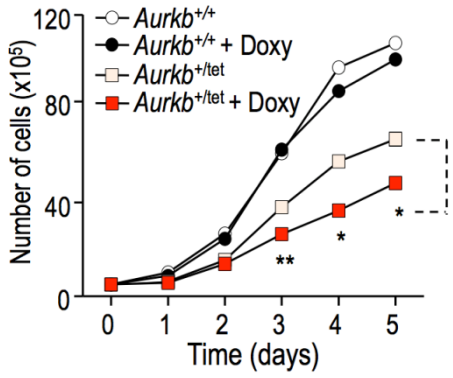


Figure 25. Growth curve analysis of Aurora B overexpressing MEFs. Proliferation of early passage primary MEFs with or without the addition of doxycycline. *Aurkb*$^{+/+}$ and *Aurkb*$^{+/tet}$ cells (always in the presence of the *Rosa26*(M2rtTA) allele) were treated with doxycycline or left untreated during five days.

2.2.3. Mitotic defects caused by Aurora B overexpression

Overexpression of Aurora B did not significantly alter the percentage of cells in mitosis, although these mutant cells displayed an increased percentage of prometaphase cells, as observed in Figure 26a, p=0.02. Mitotic defects, mostly lagging chromosomes and spindles with multiple asters, were significantly more abundant in *Aurkb*$^{+/tet}$ cells treated with doxycycline (14.4 ± 3.7 vs. 4.8 ± 2.7 in untreated cells; p=0.0245; Figure 26b and 26c). The percentage of aberrant interphasic cells, including multi-nucleated cells or cells with micronuclei (Figure 26b and 26c), was also more frequent upon Aurora B overexpression (9.8 ± 1.9 vs. 6.3 ± 0.7 in control cultures; p=0.0489).

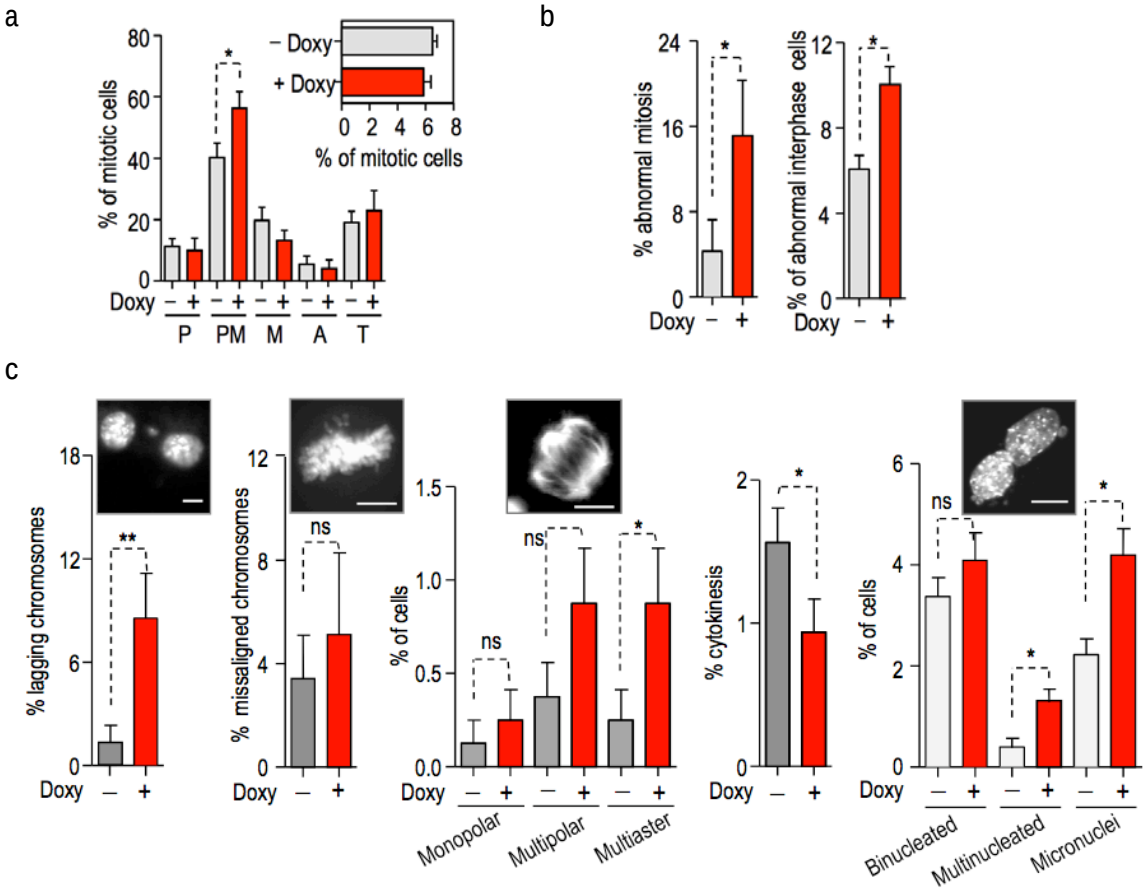


Figure 26. Mitotic defects in Aurora B-overexpressing cells. (a) Percentage and distribution of mitotic cells indicating the percentage of prometaphase (PM), metaphase (M), anaphase (A) or telophase (T) cells 48 h after the addition of doxycycline. (percentage of cells in PM: 40.34 ± 4.595 vs; 56.26 ± 5.40) (N=400 cells per treatment). (b) Percentage of abnormal mitotic figures (left panel) or abnormal interphasic cells (bi, multi and micronucleated cells, right panel) in Aurora B overexpressing cells. (N=50 cells per treatment) (c) Percentage of mitotic defects (lagging or misaligned chromosomes and aberrant spindles), cytokinesis figures and nuclear alterations in interphasic cells in *Aurkb*$^{+/tet}$ MEFS in the absence or presence of Doxycycline. Representative pictures are shown in the upper part of the graphs. DAPI was used to stain DNA. Scale bars, 5 µm. n=400 cells. ns, not significant; *, p<0.05; Student's t-test.

2.2.4. Aurora B overexpression leads to an increased duration of mitosis and loading of BubR1

The duration of mitosis was increased in Aurora B-overexpressing MEFs in the absence of these microtubule poisons (137.70 ± 9.72 min. vs. 108.2 ± 5.45 in untreated *Aurkb*$^{+/tet}$ cells; Figure 27). No differences on mitotic entry were observed in MEFs overexpressing Aurora B, but the delay in mitotic exit as shown by videomicroscopy was indicating defects in chromosome segregation (Figure 27).

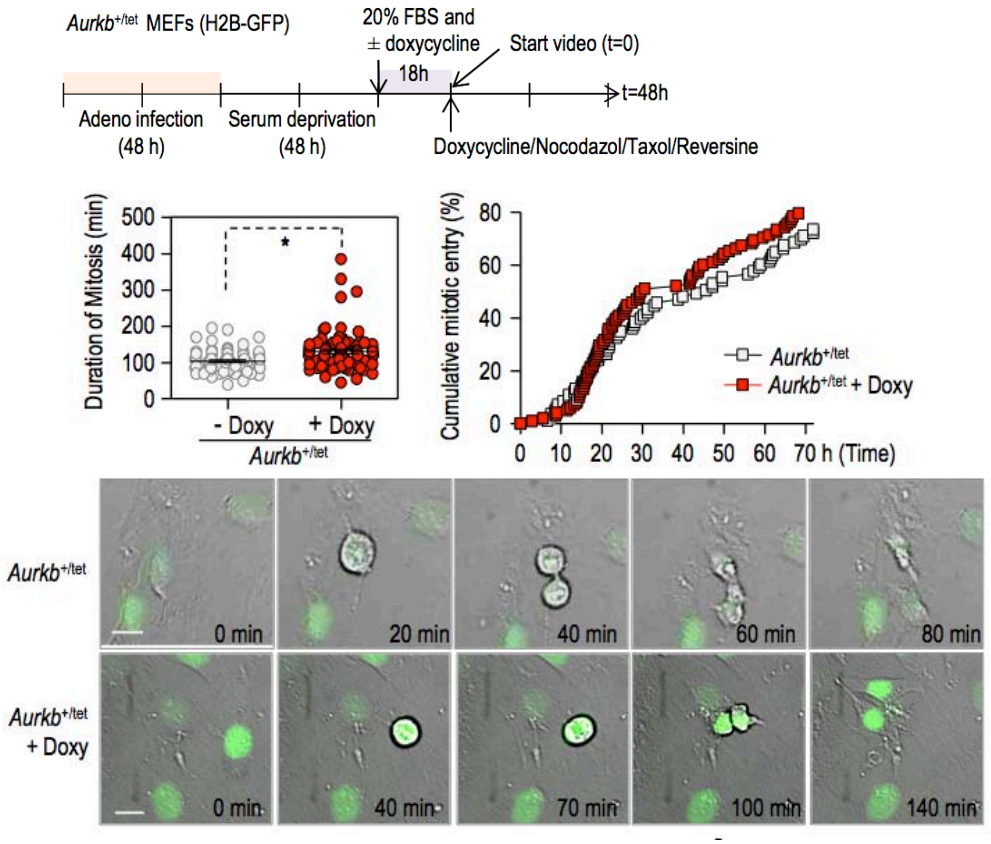


Figure 27. Increased duration of mitosis in Aurora B-overexpressing MEFs. (a) Schematic representation of the protocol used for time-lapse microscopy. Confluent cultures of immortalized *Aurkb*$^{+/tet}$; *Rosa26*$^{M2rtTA/M2rtTA}$ MEFs were infected with adenoviruses expressing GFP-tagged histone H2B (H2B-mGFP) following serum deprivation for 48 hours. These cells were stimulated with serum and treated or not with doxycycline for 18 hours. Then, the cells were left untreated or exposed to different drugs and afterwards, they were monitored by using time-lapse microscopy during an additional 48 h. (b) Duration of mitosis (minutes) in untreated or doxycycline-treated cells. The accumulation of mitotic cells (as scored by cell rounding and chromosome condensation) in these cultures is shown in the right panel. Representative images of these cultures are shown. Scale bar, 10 μm. H2B-mGFP is in green.

The defect on mitosis duration was caused by increased Aurora B as it was prevented by treatment of Aurora B overexpressing cells with the Aurora B inhibitor ZM447439 (Figure 28). In addition, the defect in the duration of mitosis was rescued by the Mps1 inhibitor, reversine (Figure 28), suggesting that mitotic delay in the presence of increased Aurora B levels was caused by SAC activation.

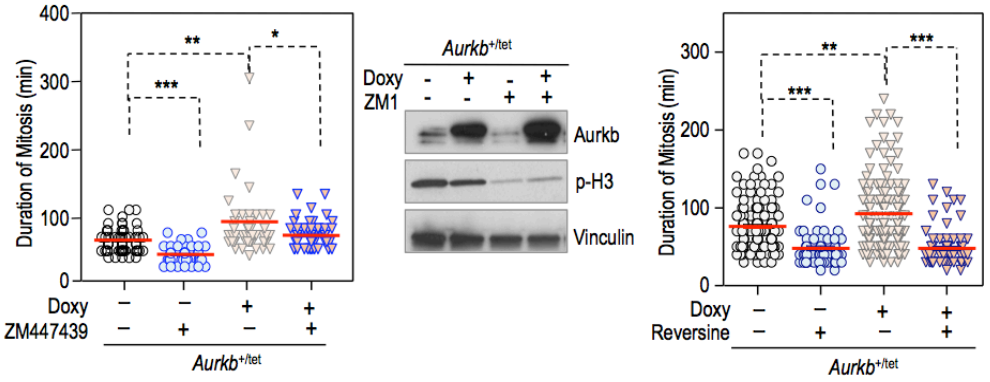


Figure 28. Increased duration of mitosis upon Aurora B overexpression is rescued by ZM447439 and reversine treatments. The delay in mitotic exit upon Aurora B overexpression was rescued by the Aurora B inhibitor ZM447439. The immunoblot shows Aurora B protein levels and phosphorylation of histone H3 (p-H3) in the absence or presence of ZM447439. Right panel shows how the delay in mitotic exit upon Aurora B overexpression was rescued by the Mps1 inhibitor reversine.

Aurora B is involved in the error correction mechanism that monitors chromosome attachments to the spindle, and its activity generates unattached kinetochores that are sensed by the SAC. Overexpression of Aurora B did not alter the response to microtubule poisons such as taxol or nocodazole in MEFs, which are known to easily exit from mitosis in the presence of these drugs (Fig. 29).

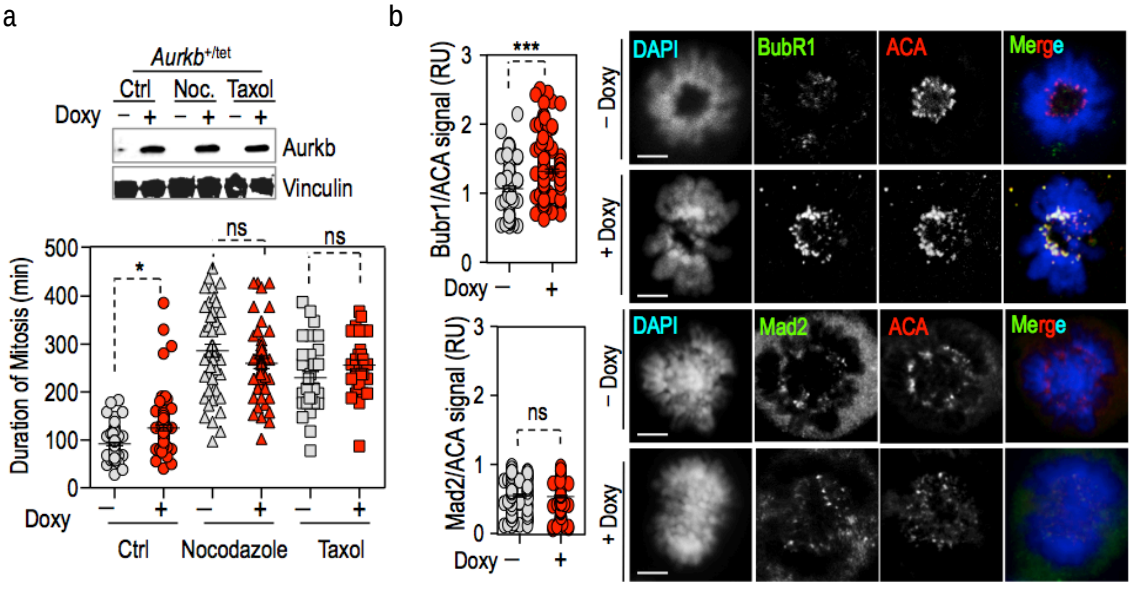


Figure 29. Aurora B overexpression results in increased duration of mitosis and increased loading of BubR1 (a) Duration of mitosis (in minutes) in immortal untreated *Aurkb*$^{+/tet}$ (N=149) or doxycycline induced *Aurkb*$^{+/tet}$ MEFs (N=164) showing an increase in the duration of mitosis when overexpressing Aurora B. No differences were found when exposed to 3.5 μM nocodazole or 1 μM taxol. (b) Ratio between BubR1and Mad2 versus anti-centromere antigen (ACA) signal in *Aurkb*$^{+/tet}$ MEFs treated or not with doxycycline. (N=at least 70 centromere signals per group). Representative images of BubR1 and Mad2 (green) staining at kinetochores in untreated or doxycycline treated *Aurkb*$^{+/tet}$ MEFs are also shown. ACA is shown in red and DNA (DAPI) in blue. Three different clones from each genotype/condition were analysed.

Whereas we found no significant differences in the association of BubR1 or Mad2 to kinetochores in the presence of microtubule poisons (data not shown), we observed a significant increase in BubR1 protein levels at centromeres in unperturbed *Aurkb*$^{+/tet}$ MEFs overexpressing Aurora B (Fig. 29). Levels of centromeric Mad2 were however unaffected in these cells, suggesting a specific limiting role for Aurora B in the kinetochore localization of BubR1.

2.2.5. Mitotic defects upon Aurora B overexpression lead to aneuploidy

In order to assess whether Aurora B overexpression induced chromosomal instability, we performed karyotype analyses of metaphase spreads generated from early (passage 2) or late (passage 30) immortal MEFs. As shown in Figure 30, overexpression or Aurora B in early passage cells resulted in a broader variance in the number of chromosomes after 5 days with doxycycline treatment. In addition, whereas control cultures were relatively stable 30 passages later, Aurora B-overexpressing cells showed a significantly wider variance in their chromosome numbers (Figure 30), as expected if there were mis-segregation of chromosomes during cell division.

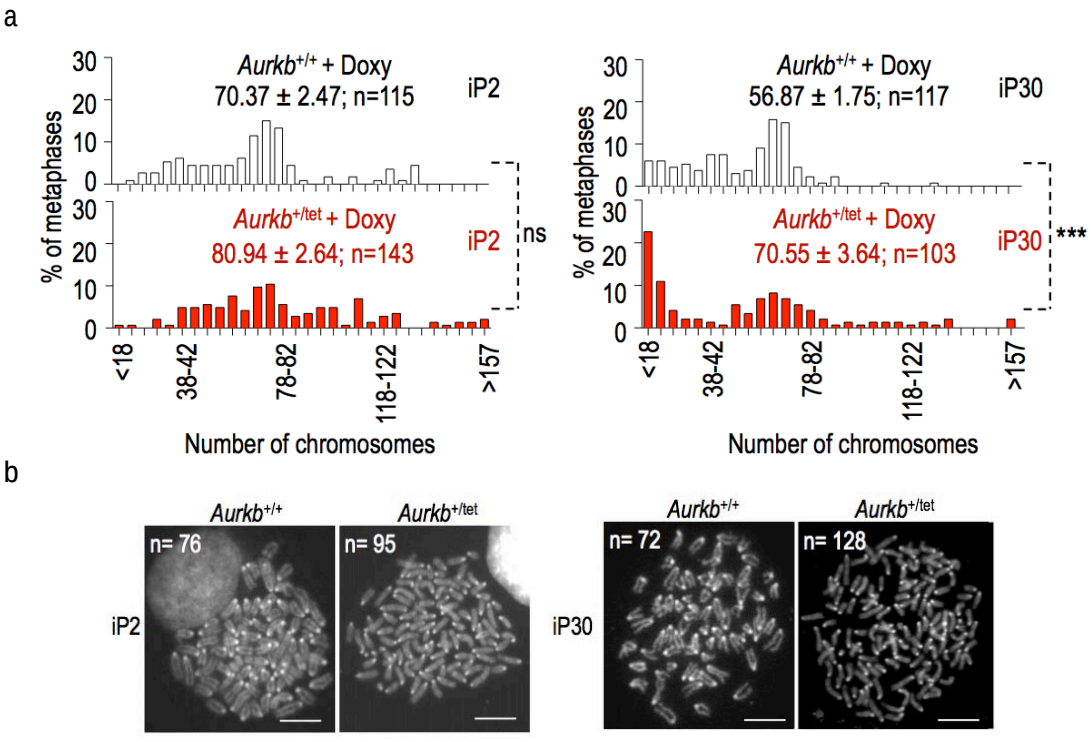


Figure 30. Overexpression of Aurora B *in vitro* leads to aneuploidy (a) Cytogenetic analysis of immortalized MEFs after exposure to 5 hours of colcemid. The absolute number of chromosomes per metaphase was grouped in categories at early (iP2) or late (iP30) immortal passages. (b) Representative images of aneuploid cells of the indicated genotypes at immortal passage (iP) 2 or 30 n represents the number of chromosomes. DNA was visualized with DAPI. Scale bar, 10 μm. ns, not significant; ***, p<0.001; Student's t-test.

2.3. Overexpression of Aurora B impairs the DNA damage response

2.3.1. Aurora B represses the expression of the cell cycle inhibitor p21^{Cip1} *in vitro* and *in vivo*

Aurora B has been shown to inactivate p53 function, lowering expression of its target genes *in vitro* (Gully et al., 2012; Wu et al., 2011). We therefore explored whether p53 or its target genes were affected by Aurora B overexpression in cells treated with the topoisomerase II inhibitor Adryamicin. The doxycycline-dependent induction of Aurora B correlated in these cells with a significant reduction in the level of induction of p21^{Cip1} protein (Figure 31a) and transcripts (Figure 31b). In these assays, the levels of p53 were not significantly affected by Aurora B overexpression (Figure 31), suggesting that Aurora B affects p53 activity rather than its protein levels. Moreover, when measuring DNA damage by H2AX staining in cells, a diminished response to this stress could be seen (Figure 31c).

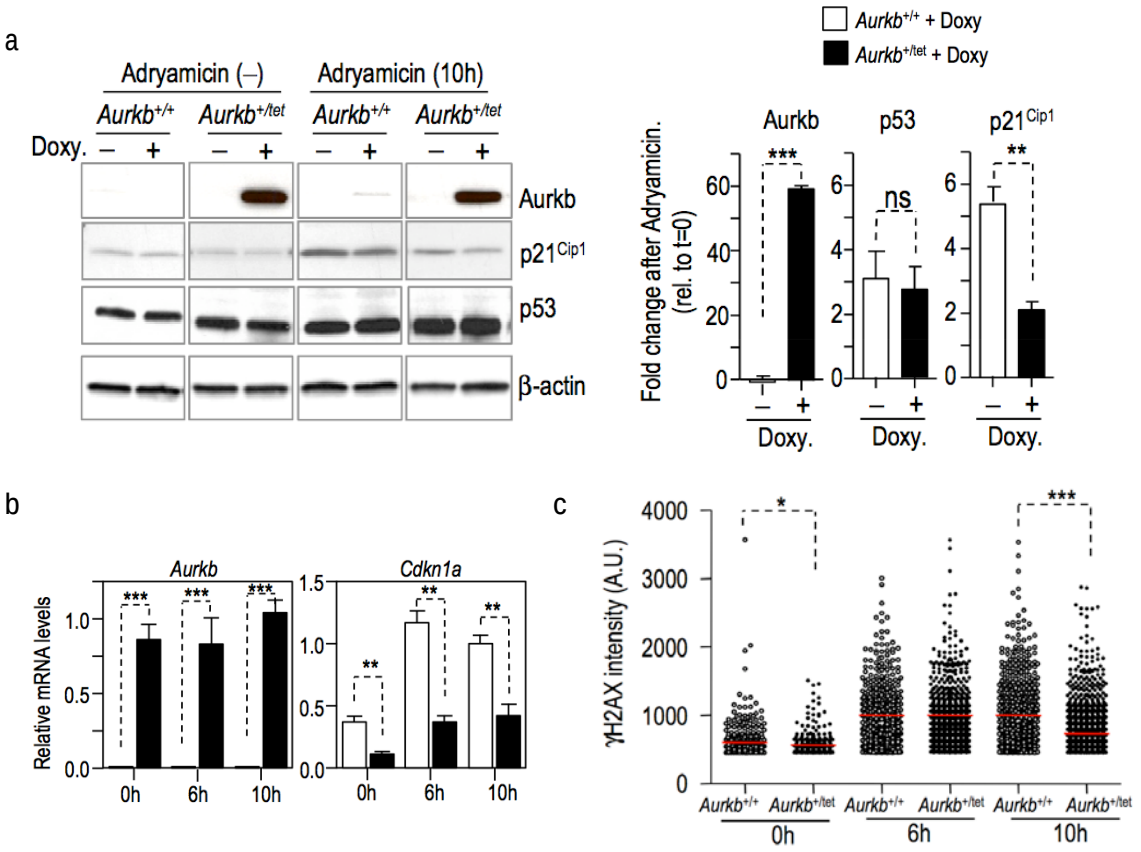


Figure 31. Overexpression of Aurora B impairs the DNA damage response *in vitro*. (a) Schematic representation of the DNA damage assay performed in *Aurkb*$^{+/tet}$; *Rosa26*$^{M2rtTA/M2rtTA}$ and*Aurkb*$^{+/+}$; *Rosa26*$^{M2rtTA/M2rtTA}$ MEFs. Cells were treated with doxycycline (Doxy) for 24 h and adryamicin was added for two hours in order to induce p53 signaling. The protein levels of the indicated proteins were assessed before and ten hours post-damage induction and the fold change versus non-damaged cells is shown on the right histograms. (b) mRNA levels of *Aurkb* and *Cdkn1a* (encoding p21^{Cip1}) transcripts in *Aurkb*$^{+/tet}$; *Rosa26*$^{M2rtTA/M2rtTA}$ and *Aurkb*$^{+/+}$; *Rosa26*$^{M2rtTA/M2rtTA}$ MEFs at the indicated times (0, 6 and 10 h) after treatment with adryamicin. *Gadph* transcripts were used for normalization. (c) γH2AX levels in cells overexpressing Aurora B were significantly reduced after 10 hours of being treated with adryamicin ns, not significant; *, p< 0.05, **, p<0.01; ***, p<0.001; Student's t-test.

We next analysed whether Aurora B regulates the p53 pathway *in vivo* by treating *Aurkb*$^{+/+}$ and *Aurkb*$^{+/tet}$ mice, in the presence of the Rosa26$^{M2rtTA/M2rtTA}$ alleles, with γ- irradiation (8 Gy). The intensity of Aurora B immunostaining increased in the white pulp of the spleen after treatment with doxycycline both in irradiated and non-irradiated mice (Figure 32). Whereas overexpression of Aurora B did not significantly alter the induction of p53 in these irradiated mice, the percentage of p21^{Cip1} positive cells was significantly reduced in irradiated *Aurkb*$^{+/tet}$ mice compared to wild-type controls (Figure 32). This effect was accompanied by a reduced response to DNA damage, as scored by phosphorylation of H2AX (γH2AX), and a significant decrease in the ratio of apoptotic cells after irradiation (Figure 32), suggesting that overexpression of Aurora B also impairs the DNA damage response *in vivo*.

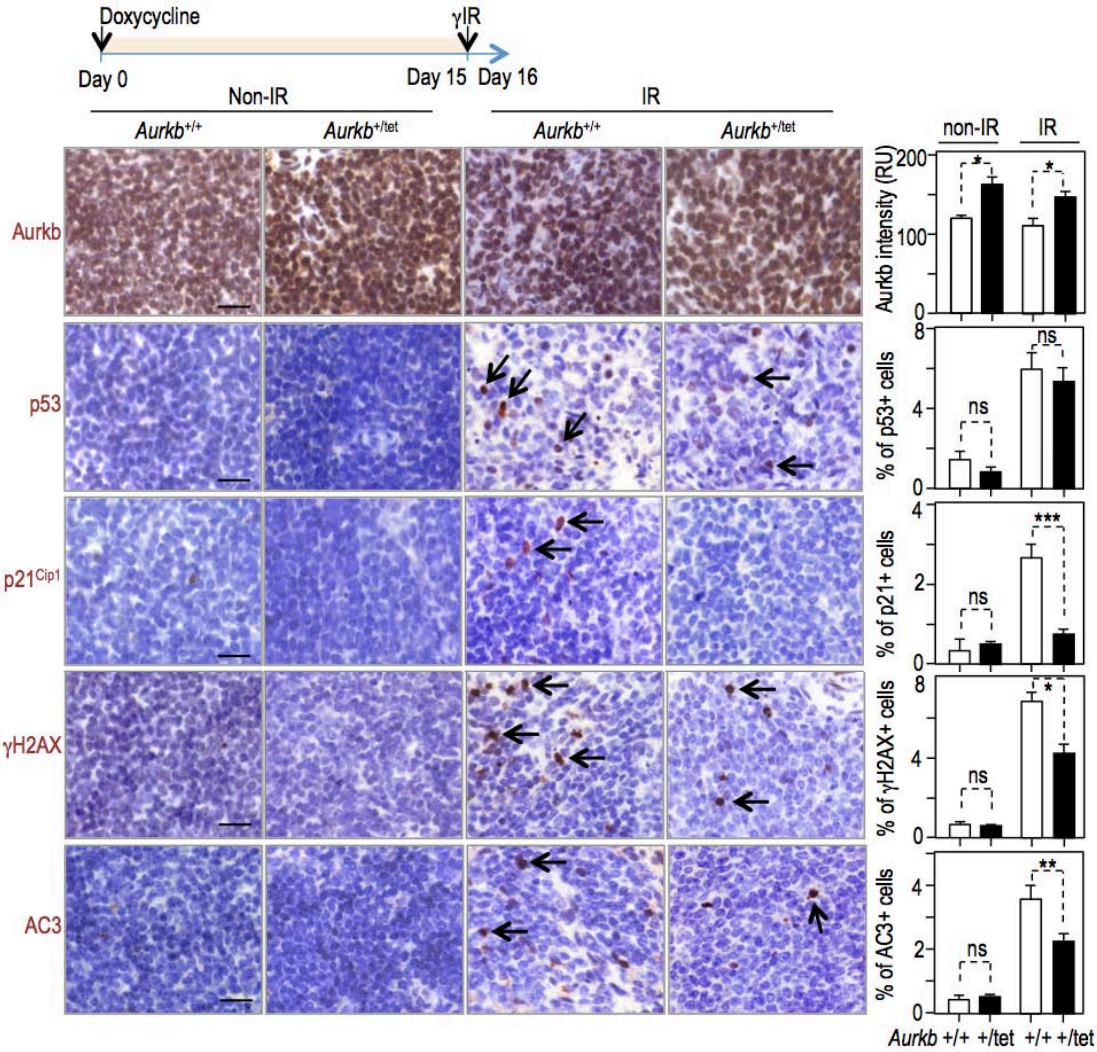


Figure 32. Overexpression of Aurora B impairs the DNA damage response *in vivo*. (b) To induce DNA damage *in vivo*, 6-8-week-old *Aurkb*$^{+/+}$; *Rosa26*$^{M2rtTA/M2rtTA}$ mice and *Aurkb*$^{+/tet}$; *Rosa26*$^{M2rtTA/M2rtTA}$ mice were treated for fifteen days with doxycycline and were subjected to irradiaton (IR; 8 Gy γ-irradiation). The level of the indicated proteins was tested by immunohistochemistry in the spleen 24 h later. AC3, active caspase 3. Scale bar, 50 μm. ns,not significant; *, p<0.05; **, p<0.01; ***, p<0.001; Student's t-test.

2.4. Aurora B overexpressing mice are tumour prone and aneuploid

2.4.1. Increase tumour incidence in Aurora B overexpressing mice

To monitor long-term effects of Aurora B overexpression *in vivo*, Rosa26$^{M2rtTA/M2rtTA}$ mice harboring the mutant *Aurkb(*tet) or *Aurkb(*loxtet) alleles were treated with doxycycline in the drinking water for 20 months. We first confirmed the *in vivo* induction of Aurora B by analyzing mRNA (Figure 33a) and protein levels (Figure 33b) in several proliferative and non-proliferative tissues of Aurora B overexpressing mice. Although both mutant alleles resulted in Aurora B overexpression, the levels of Aurora B were higher in *Aurkb*$^{+/tet}$ tissues when compared to the corresponding *Aurkb*$^{+/loxtet}$ samples.

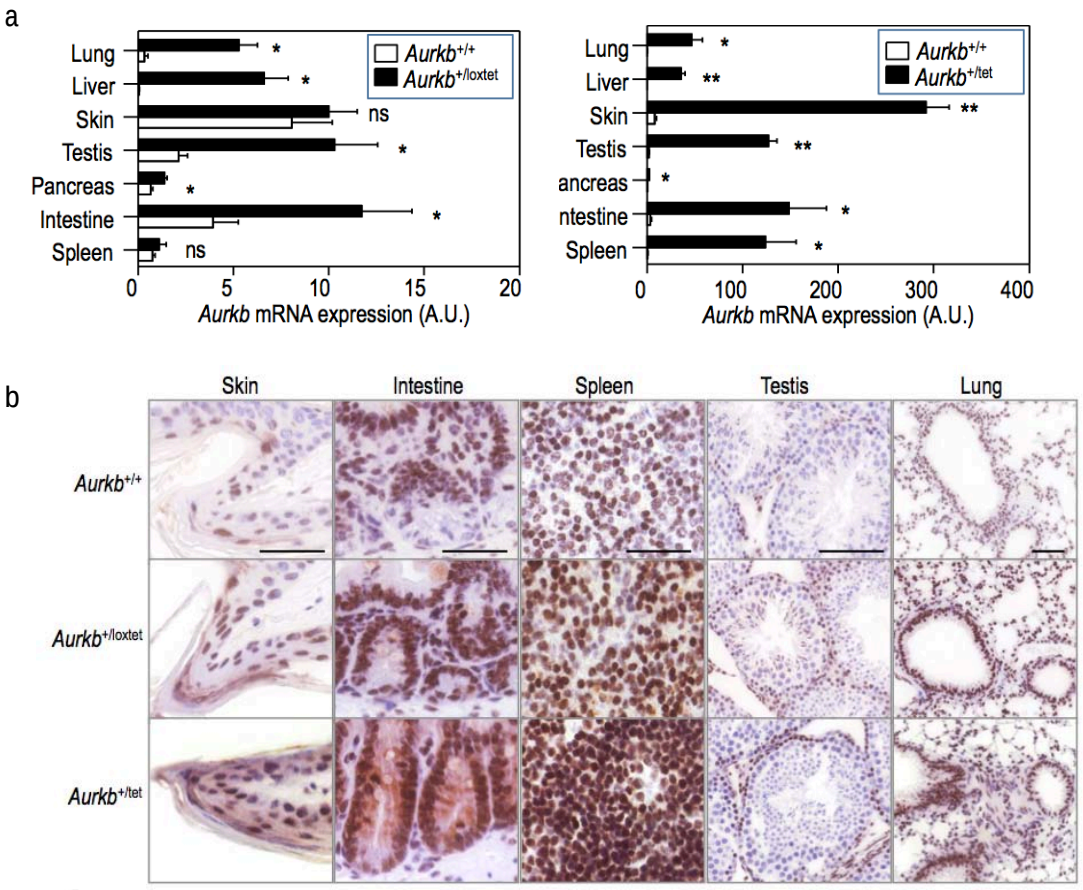


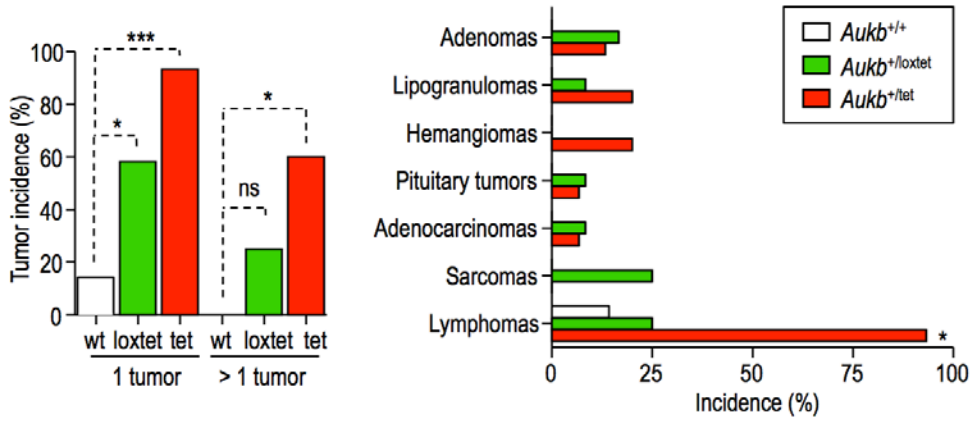
Figure 33. *In vivo* characterization of Aurora B overexpressing mice. (a) Expression of Aurora B transcripts in the indicated tissues from *Rosa26*$^{M2rtTA/M2rtTA}$ mice harboring the *Aurkb(*loxtet) and *Aurkb(*tet) alleles after treatment with doxycycline for two weeks. mRNA levels were normalized versus the expression of *Gadph* mRNA. (b) Immunohistochemical detection of Aurora B in the indicated tissues and genotypes. Scale bar, 50 μm.

Whereas for Mad2 and Bub1, the relationship between them and tumour initiation has been precisely explored, whether Aurora B has a role in aneuploidy induction and tumour development still remains unclear. Some studies have related Aurora B deregulation to aneuploidy and cancer (Terada, 1998; Ota, 2002; Nguyen, 2009) and its oncogenic potential has been measured together with Ras

oncogene (Kanda *et al;* 2005), but the tumourigenesis potential of Aurora B and the molecular and cellular consequences of Aurora B overexpression have not been proven by itself.

Importantly, *Aurkb*$^{+/tet}$ and *Aurkb*$^{+/loxtet}$ mice were characterized by a high incidence of tumour development during ageing. Whereas spontaneous tumours were detected in 14.28% of control mice, 58.3% of the *Aurkb*$^{+/loxtet}$ mice and more than 90% *Aurkb*$^{+/tet}$ mice had at least one tumour (p=0.0006 *Aurkb*$^{+/tet}$ vs. wild-type mice; Figure 34a). Furthermore, 60% of *Aurkb*$^{+/tet}$ mice and 25% of *Aurkb*$^{+/loxtet}$ mice were simultaneously affected with more than one tumour type (Figure 34a). Histological analysis of both *Aurkb*$^{+/tet}$ and *Aurkb*$^{+/loxtet}$ mice showed a wide spectrum of tumours, with spleen lymphomas being the most common neoplasia (25% and 93.3% of *Aurkb*$^{+/loxtet}$ and *Aurkb*$^{+/tet}$ mice, respectively; Figure 34b).

a



b

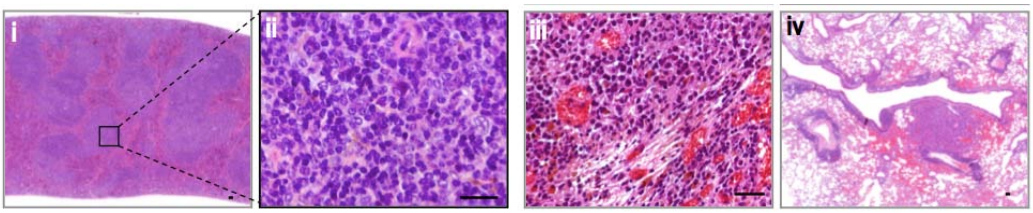


Figure 34. Aurora B overexpressing mice develop spontaneous tumours. (a) Spontaneous tumour incidence (one tumour or more than 1 tumour per mouse) (b) Type of neoplasias found in Aurora B-overexpressing mice (percentage of incidence is represented). (c) Hematoxylin and eosin (H&E) staining of the indicated tumours found in *Aurkb*$^{+/tet;}$ *Rosa26*$^{M2rtTA/M2rtTA}$ mice. i,ii, spleen lymphoma; iii, histiocytic sarcoma; iv, lung adenoma. ns, not significant; *, p<0.05; ***, p<0.001; Chi-Square test. Scale bar, 100 µm:

Aurora B overexpression in Aurkb$^{+/tet}$ mice also resulted in sarcomas (25%), alveolar type II cell adenomas in the lung (13.33%), liver hemangiomas (20%) as well as other more infrequent tumours and non-tumoural lesions (Figure 34).The lymphomas found in Aurora B overexpressing mice were follicular B cell subtype (Pax5-positive; Figure 35) characterized by enlarged spleen follicles populated by a mixture of cells including centrocytes, centroblasts and Cd3+ T-cells (Figures 34 and 35).

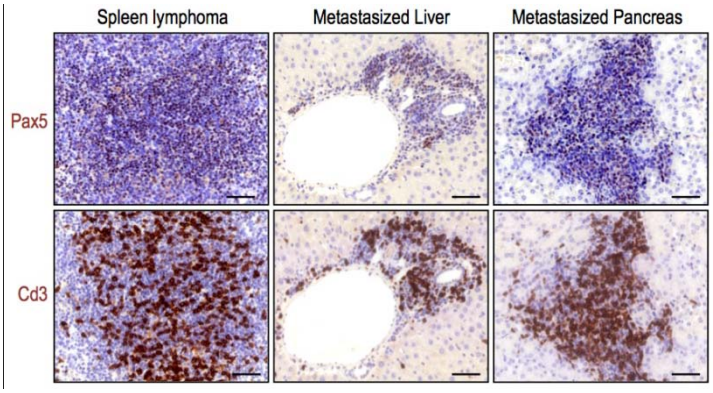


Figure 35. Characterization of spleen lymphomas found in Aurora B overexpressed mice. Representative pictures of Pax5 and CD3 staining in tumour samples from the spleen, liver and pancreas of *Aurkb*$^{+/tet;}$ *Rosa26*$^{M2rtTA/M2rtTA}$ mice. Scale bar, 50 µm.

Aurkb$^{+/tet}$ lymphomas presented a higher degree of aggressiveness as shown by infiltrations in the lung, liver, kidney and bone marrow, where these tumours preferentially metastasized (Figure 36).

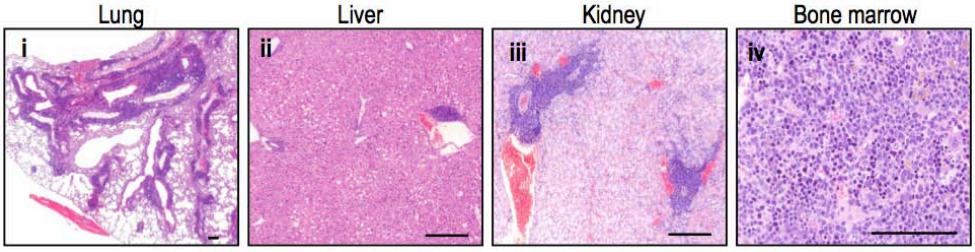


Figure 36. Metastatic tumours in Aurora B overexpressing mice. H& E images of lymphoma metastasis in the lung, liver, kidney and bone marrow of *Aurkb*$^{+/tet;}$ Rosa26$^{M2rtTA/M2rTTA}$ mice. Scale bar, 200 µm.

2.4.2. Aneuploidy in Aurora B overexpressing mice

We next asked whether overexpression of Aurora B caused aneuploidy in peripheral blood lymphocytes of mutant mice. We scored interphasic nuclei by FISH, using probes against chromosomes 8 and 11. Age-dependent accumulation of aneuploidy in lymphocytes was observed in both genotypes although *Aurkb*$^{+/tet}$ animals contained significantly higher percentage of aneuploid cells (Figure 37). The percentage of aneuploidy, calculated as the deviation from the mode, was 0.28% in 4-month old *Aurkb*$^{+/+}$ mice and 3.04% in *Aurkb*$^{+/tet}$ littermates (p=0.0021). At 20 months of age, these numbers increased to 3.00% in *Aurkb*$^{+/+}$ and 7.66% in *Aurkb*$^{+/tet}$ mice (p= 0.0033; Figure 37). Levels of aneuploidy were not significantly different when analyzing untreated *Aurkb*$^{+/+}$, *Aurkb*$^{+/loxtet}$ or *Aurkb*$^{+/tet}$ mice aged for 20 months.

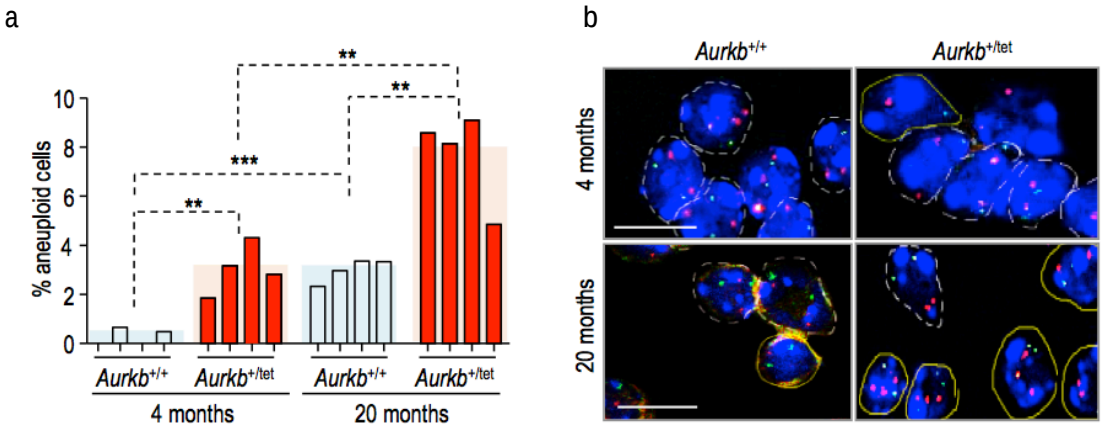


Figure 37. *In vivo* overexpression of Aurora B induces aneuploidy. (a) Levels of aneuploid cells in *Aurkb*$^{+/+}$; *Rosa26*$^{M2rtTA/M2rtTA}$ and *Aurkb*$^{+/tet}$;*Rosa26*$^{M2rtTA/M2rtTA}$ mice after 4 or 20 months in the presence of doxycycline. Aneuploidy (deviation from the mode) was scored using FISH for two different chromosomes in lymphocytes from 4 and 20-month-old animals (at least n=100 from each of four animals per genotype and time point). Each column represents one animal of the indicated genotypes. **,p<0.01; ***, p<0.001; Student's t-test. (b) Representative images of interphasic FISH analysis using probes for chromosomes 8 (red) and 11 (green).Dot lines represent aneuploid cells. Scale bar indicates 20 μm.

2.5. Aurora B overexpressing tumours display increased aneuploidy and reduced p21 ^{Cip1} levels

2.5.1. Increased aneuploid levels in Aurora B overexpressing tumours

Spleen sections from tumour-bearing and healthy 20-month-old *Aurkb*$^{+/tet}$ and wild-type mice were hybridized with FISH probes against chromosomes 8 and 11. The percentage of aneuploid cells in non-metastatic ("initial") or advanced, metastatic *Aurkb*$^{+/tet}$ tumours was significantly higher than in non-tumoural (NT) spleen cells from age-matched wild-type animals (Figure 38a). Aneuploidy also increased in the spontaneous tumours observed in *Aurkb*$^{+/+}$ mice (8% of aneuploid cells in spleen tumours versus 4% in NT spleens), although it never reached the levels found in initial (10.9%) or advanced (14.8%) Aurkb$^{+/tet}$ tumours (Figure 38).

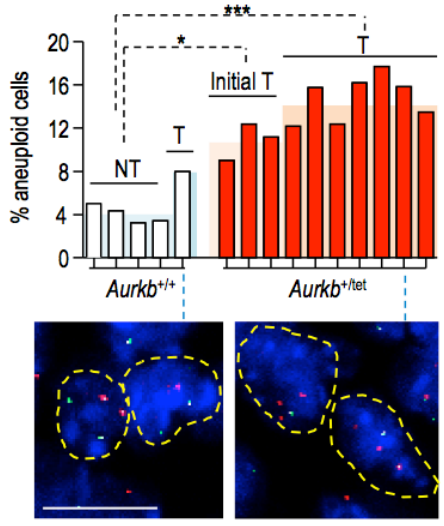


Figure 38. Aurora B overexpressing mice develop spontaneous tumours with aneuploid cells. Percentage of aneuploid cells in non tumoural (NT) spleens, or spleens with initial or fully developed tumours (T) from the indicated genotypes. Initial tumour refers to small, non-metastatic tumours. FISH was performed for chromosomes 8 (red) and 11 (green) on spleen sections from 80-week old mice.Dot lines respresent aneuploid cells. Each column represents one animal of the indicated genotypes. Representative images are shown at the bottom. Scale bar indicates 20 μm. At least 100 cells were counted per condition.

The increase in total aneuploidy reflected both an increase in both near-diploid and near-tetraploid aneuploid cells within spleen lymphomas (Figure 39). Similar results were found in lung tumours, the most prominent epithelial-originated tumour in Aurkb+/tet mice. Together these data suggest that Aurora B-overexpression leads to chromosomal instability *in vivo*, which may precede to overt transformation.

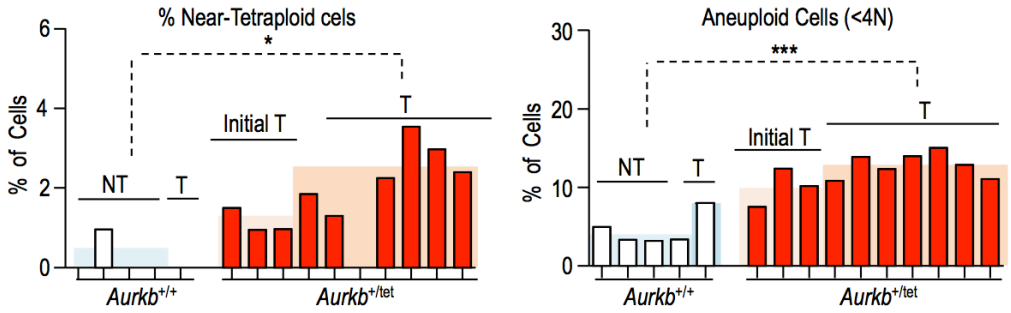


Figure 39. Aneuploidy levels in spleens from Aurora B overexpressed mice. Percent of near-tetraploid cells and aneuploid cells (excluding those scored as near-tetraploid) determined by chromosome FISH hybridization in non-tumoural (NT) or tumoural [(T), either initial (Init-T) or fully developed tumours (T)] spleen samples from *AurkB*$^{+/+;}$ *Rosa26*$^{M2rtTA/M2rtTA}$ and *AurkB*$^{+/tet;}$ *Rosa26*$^{M2rtTA/M2rtTA}$ mice. Each column represents one animal of the indicated genotypes.

2.5.2. Reduced p53 and p21 levels in Aurora B overexpressing tumours

By monitoring the doxycycline treated animals we observed that the Aurora B overexpresing mice are more prone to develop spontaneous lymphomas and histiocytic sarcomas and present a shortening life span versus the wild type mice. Moreover, since Aurora B can modulate p21^{Cip1} levels (Figure 31), we also analysed p53 and p21^{Cip1} levels in spleen tumours. p53 protein levels were significantly reduced in *Aurkb*$^{+/tet}$ tumours in comparison to tumoural or non-tumoural spleen samples from *Aurkb*$^{+/+}$ mice (Figure 40). Similarly, a significant decrease in p21^{Cip1} levels was also found in spleen tumours from Aurkb$^{+/tet}$ mice compared with similar tumours from *Aurkb*$^{+/+}$ animals (Figure 41a). p21^{Cip1} was almost absent in metastatic *Aurkb*$^{+/tet}$ tumours, in line with their aggressive phenotype.

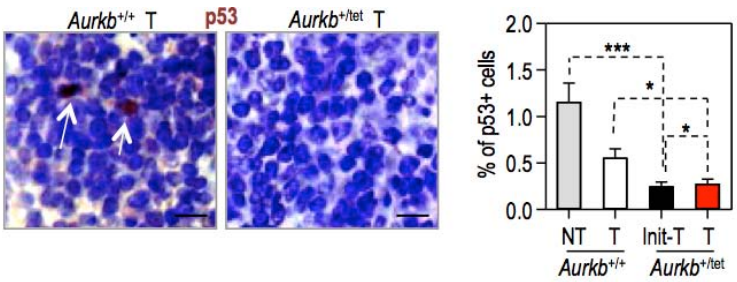


Figure 40. p53 levels in non-tumoural and tumoural spleen samples from overexpressing Aurora B mice. Immunodetection of p53 in non-tumoural (NT) or tumoural (T; either initial or fully developed) spleen samples from *AurkB*$^{+/+;}$ *Rosa26*$^{M2rtTA/M2rtTA}$ and *AurkB*$^{+/tet;}$ *Rosa26*$^{M2rtTA/M2rtTA}$ mice. Scale bar, 80 µm. The quantification of the percentage of p53 positive cells is shown in the histogram. *, p<0.05; ***, p<0.001; Student's t-test.

To test whether these observations may also apply to human lymphoblastic leukaemias, we compared *AURKB* and *CDKN1A* (the human gene encoding p21Cip1) mRNA levels in B acute lymphoblastic leukaemia (B-ALL) samples (Harvey et al., 2010; Kang et al., 2010). As represented in Figure 41b, the levels of expression of these transcripts displayed a significant inverse correlation in which $p21^{Cip1}$ expression was constantly low in tumours expressing high levels of Aurora B. All together, these data suggest that Aurora B overexpression triggers tumour development in association with both increased aneuploidy and defective $p21^{Cip1}$ function.

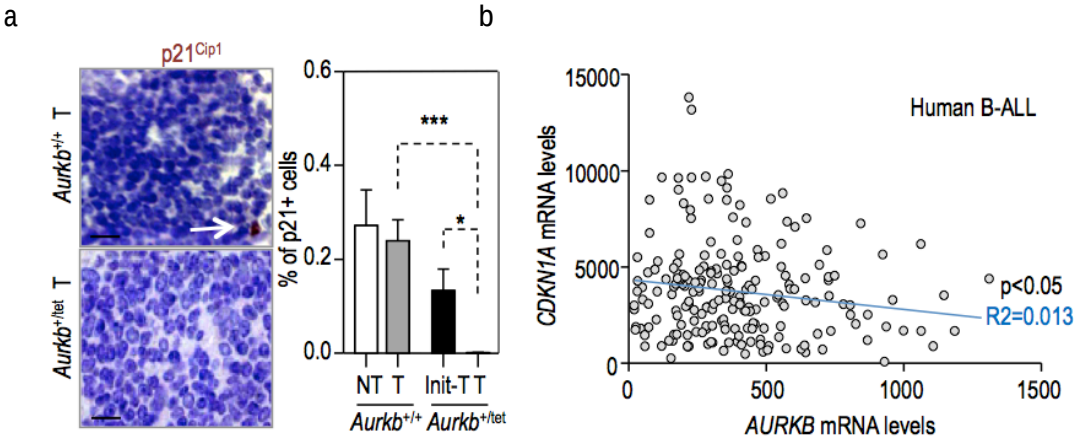


Figure 41. Aurora B overexpression correlates with $p21^{Cip1}$ repression. (a) $p21^{Cip1}$ levels in nontumoural (NT) and tumoural (T) spleen samples from *Aurkb*$^{+/+}$ mice in comparison to initial and fully developed tumours from *Aurkb*$^{+/tet}$ mice. Scale bar, 80 µm. *, p<0.05; ***p<0.001; Student's t-test. (b) Inverse correlation between *AURKB* and *CDKN1A* mRNA levels in 207 samples of human B acute lymphoblastic leukaemia (B-ALL). Pearson correlation; p<0.05. The expression of *Aurkb* mRNAs in human tumours was analysed using the Oncomine database (Compendia Bioscience). The number of analyses with significant (P < 0.0001, Student t test; fold change, >2; gene rank threshold, top 10%) overexpression of each gene is indicated.

2.6. Aurora B overexpression results in a metabolic defect in adult mice

Interestingly, since 7 months of doxycycline treatment, we observed in *Aurkb*$^{+/tet}$ mice an increase in their body weight accompanied by huge liver, spleen and pancreas steatosis and big visceral fat vacuoles surrounded by lymphocytes (Figure 42). Furthermore, a mix of brown adipose tissue and white adipose tissue (BAT/WAT) was observed in particular areas of the BAT (Figure 42). This phenotype led us to think on an Aurora B role in metabolism. In fact, it has been previously describe that Aurora B has an important role in the transcription initiation of T3-responsive genes by interaction with tyroid receptor (T3) in a ligand dependent manner in cells that do not proliferate (Tardáguila et al., 2011).

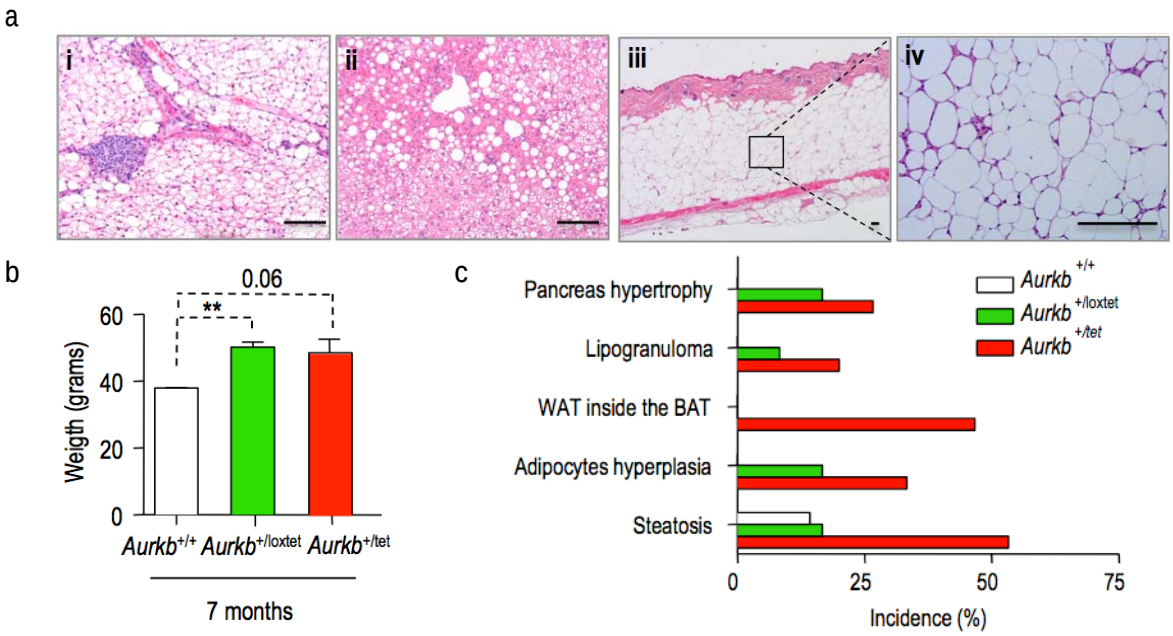


Figure 42. Metabolic alterations found in Aurora B overexpressing mice. (a) Hematoxylin and eosin (H&E) staining of the indicated tissues from *Aurkb*$^{+/tet;}$ *Rosa26*$^{M2rtTA/M2rtTA}$ mice. i, mixed BAT and WAT; ii, liver steatosis; iii and iv, fat vacuoles. Scale bar, 100 µm.(b) *Aurkb*$^{+/loxtet}$ treated with doxycycline showed a significant gain of weight when compared with control mice (**, p< 0,01; n=3) (c) *Aurkb*$^{+/tet}$ mice were characterized by different pathologies, being steatosis and mixed BAT& WAT the most prominent.

Then, we decided to analyse the cause of this surprising phenotype by taking advantage of a metabolic cage (Oxylet machine). *Aurkb*$^{+/tet}$ mice and *Aurkb*$^{+/+}$ mice were subjected to that analyse after 1.5 months after doxycycline treatment showing altered calorimetry and activity. By analyzing the respiratory quotient (RQ) value, an indirect form of calorimetry, which is the ratio C_{02} eliminated/ O_2 consumed, a reduced metabolic flexibility could be observed. And from these parameters we studied the energy expenditure (EE), which refers to the amount of energy (calories) used by the body, a reduced value was obtained in the Aurora B overexpressing mice. Regarding the intake, the water intake was equal in Aurora B overexpressed and wild-type mice whereas there was a tendency in the food intake to be higher in the transgenic mice as well as in their activity, though none of these parameters was statistically significant (Figure 43). Since a tendency in several of these parameters exits, we will further follow the analysis of the Auorora B overexpressing mice until 6 months of doxycycline treatment, time at which the apparent metabolic phenotype could be observed.

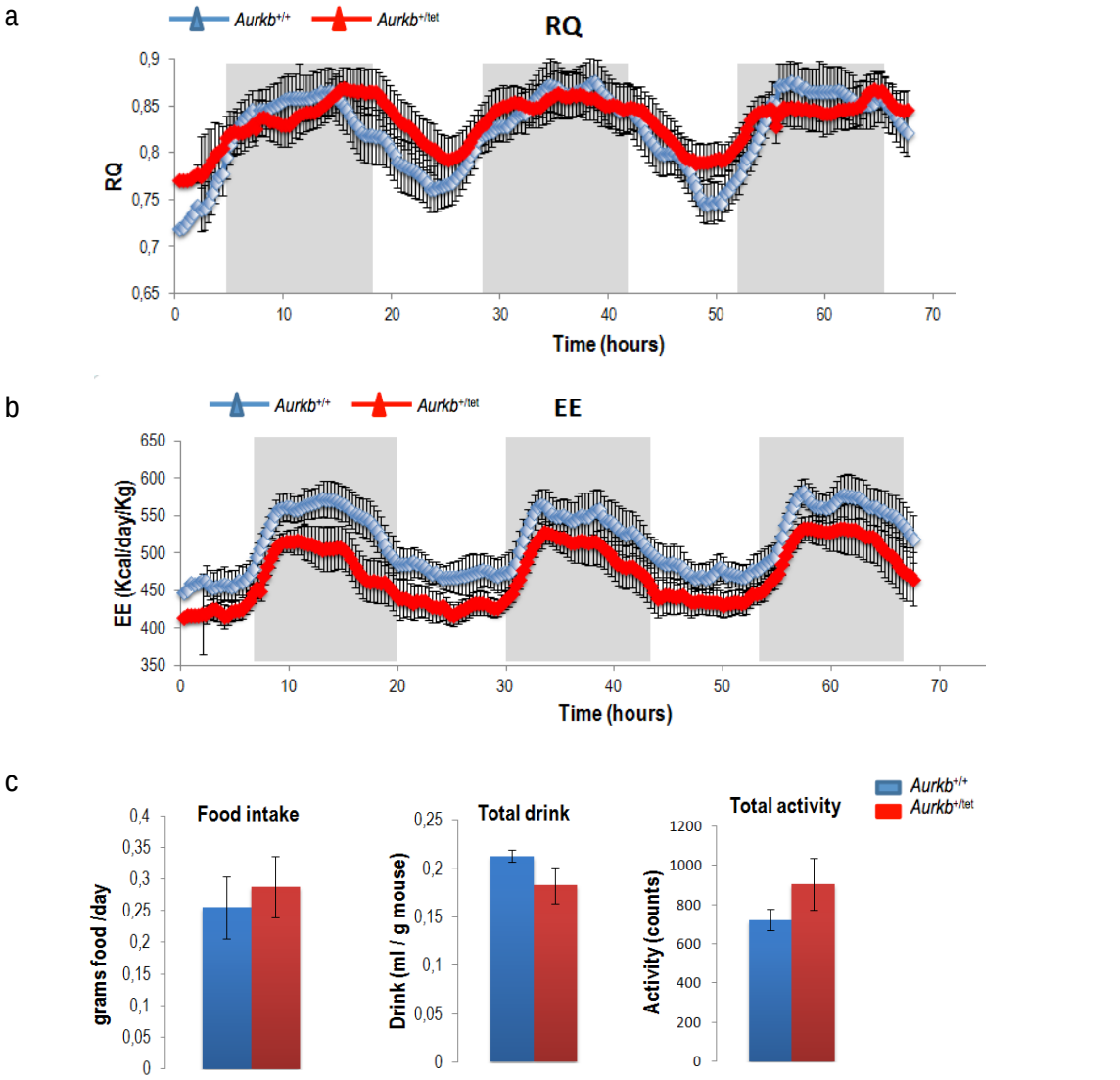


Figure 43. Metabolic parameters analysed in Aurora B overexpressing mice. (a) RQ value (Vo2/Vco2) in wild- type and Aurora B overexpressing mice during day and night (after 1.5 months of doxycycline treatment). (b) Energy expenditure (calculated from the V02 and VCO2 using the Weir Equation;p=0.094. (c) Total Food and drink intake and total Activity in wild-type and transgenic mice. Night hours are highlighted in grey. ANCOVA's test; n.s.

3. Differential role of Aurora C and B in pluripotency induction

The use of pluripotent cells to understand human disease is crucial nowadays. Induced pluripotent cells (iPSCs) are manifested towards an 'open' and dynamic chromatin state that enables their functional plasticity, which is thought to be ensured by epigenetic marks transmitted through cell division. We previously observed that Aurora C is specifically expressed in early embryos/embryonic stem cells, whereas at the blastocyst stage this kinase is replaced by Aurora B (except germ cells; (Fernández-Miranda et al., 2011). Moreover, the study of Sabbattini P. and collaborators (Sabbattini et al., 2007) saw that Aurora B epigenetically marks silent chromatin during cell differentiation. Taking into account these

data we hypothesize that Aurora C may have opposed regulatory functions to Aurora B, so that during reprogramming to pluripotency, Aurora C could enhance access to chromatin by epigenetic modifications (Figure 44). Since no experiments to date have addressed what is the endogenous function of Aurora C in any somatic cell, we aim to discover how this protein works and the best way to analyse this objective is in embryonic stem cells (ESs) and induced pluripotent cells (iPCs).

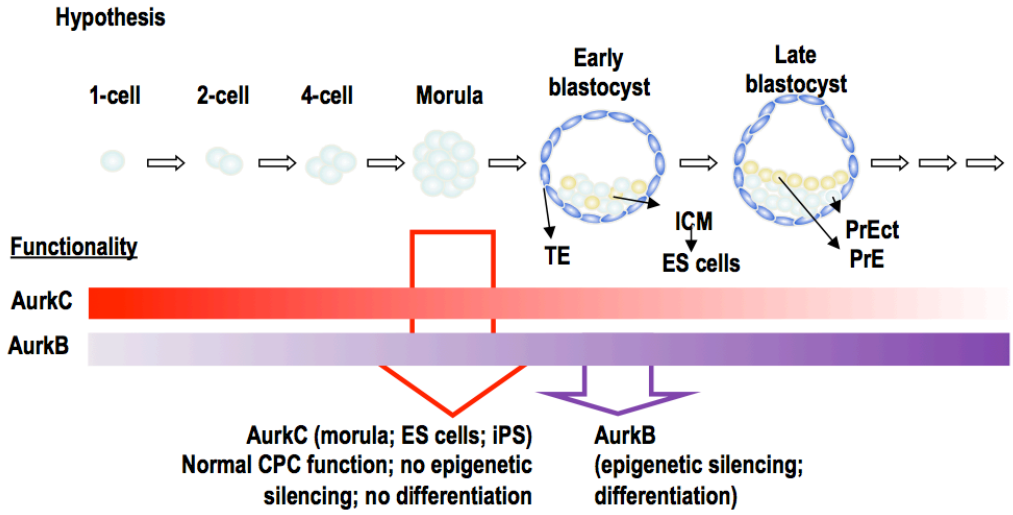


Figure 44. Hypothesis on the role of Aurora C in pluripotency. Aurora B levels increase while embryo development progresses, whereas Aurora C levels are high till the morula stage where it functions as the CPC kinase and decrease during subsequent embryo divisions. This data together with the fact that Aurora B seems to be implicated in differentiation processes by epigenetically silencing chromatin domains led us think that Aurora C may have a role in mediating stemness function.

3.1. Aurora C is not expressed in MEFs but it is activated during reprogramming to iPCs

We focused on studying the functional differences between these two important kinases, Aurora B and C. First, we corroborated that both iPSCs and ES express Aurora C at the transcript level (Figure 45a). Since no good antibody for Aurora C is available its protein levels could not be analysed. As expected, the protein levels of Aurora B and A were much higher in ESs and iPCs than in MEFs as it was for the phosphorylation of histone 3 (Figure 45b).

By taking advantage of doxycycline inducible 4F MEFs (Abad et al., 2013), carrying the transcriptional activator (rtTA) within the ubiquitously-expressed *Rosa26* locus and a single copy of a lentiviral doxycycline-inducible polycistronic cassette encoding the four murine factors *Oct4, Sox2, Klf4*and *c-Myc*, the endogenous levels of Aurora B and C were studied during the reprogramming of murine cells to iPCs. Endogenous Aurora C expression was clearly increased during reprogramming at the same time that Nanog increased (day 6 after the initiation of the reprogramming process), whereas Aurora B expression was maintained equal throughout the reprogramming to iPCs (Figure 45c).

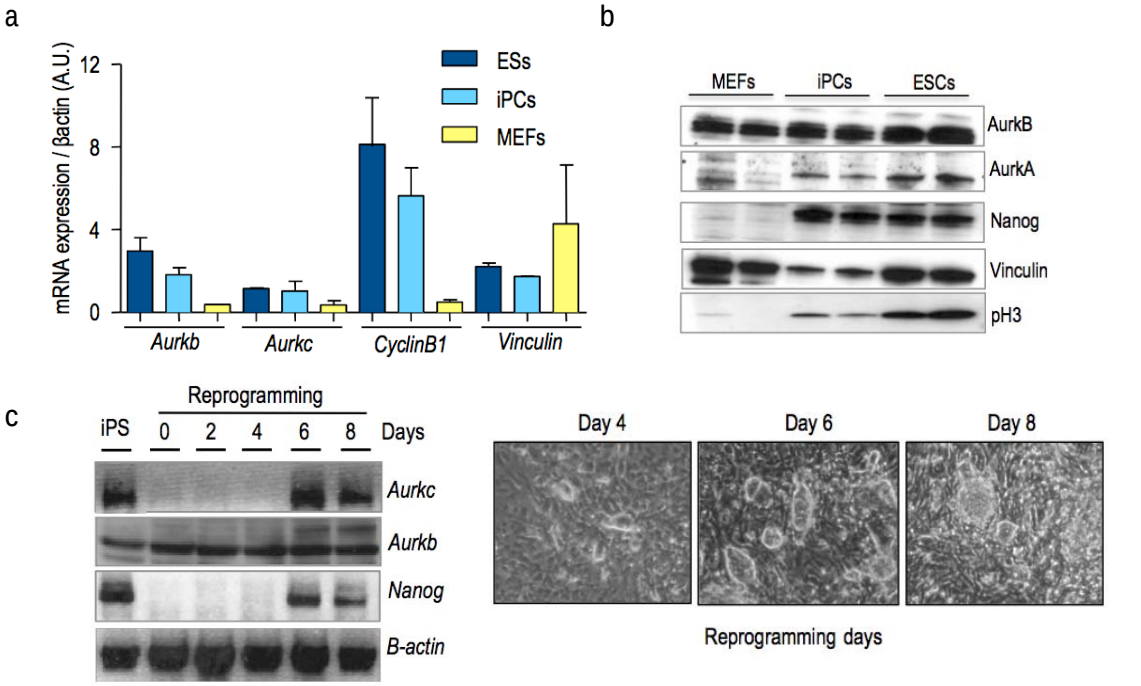


Figure 45. Aurora C is expressed in iPCs and ES cells and is activated during the reprogramming process. (a) Trancript levels of Aurora B and C in ESs, iPCs and MEFs. Cyclin B1 was used as an internal control of cell division as it was vinculin as a marker characteristic of fibroblasts. (b) Inmunoblot analysis in MEFs, iPCs and ES cells showing an upregulation of Aurora kinases A and B and pH3 in ESCs and iPCs versus MEFs. Nanog was used as a control of pluripotency and vinculin as being expressed in cell to cell adhesion (MEFs) and cell to matrix (ESCs mainly). (c) RT-qPCR analysis of Aurora kinases B and C mRNA levels were measured by q-PCR during the reprogramming process in doxycycline inducible OSKM MEFs which shows no differences in the levels of Aurora B but an increase in the levels of Aurora C starting at day 6 of the reprogramming process. Nanog was used as a control of pluripotency and β-actin as a loading control. On the right, representative pictures of primary mouse embryonic fibroblasts (MEFs) during reprogramming to pluripotent cells, showing pluripotent colonies Rosa-rtTA; lenti-4F MEFs were used to induce pluripotency conditionally by adding 1µg/ ml doxycycline to cells media. iPS RNA extract was used as control of the experiment.

3.2. Overexpression of Aurora C enhances the efficiency of reprogramming to iPCs

Then, we focus our study on analyzing the reprogramming efficiency upon Aurora B/C overexpression/depletion. We used murine embryonic fibroblasts that were transfected one day before pluripotency induction with plasmids expressing GFP (empty vector,EV) or GFP fusion proteins containing wild-type Aurora C wt or Aurora B wt, or kinase dead (KD) mutant forms of these proteins, Aurora C-KD, Aurora B-KD. These primary cells were reprogrammed and stained for Alkaline Phosphatase at day 0, 3, 6, 9, 12 and 15 in order to count the number of stem cell colonies.

We clearly visualized a highly significant enhancement (3.67 times fold change) of the reprogramming efficiency (number of ES colonies) upon Aurora C overexpression when compared to Aurora B or empty vector conditions. And as expected in the KD forms of both proteins, the number of colonies was significantly reduced (Figure 46). We can conclude that Aurora C and not Aurora B seem to be necessary for maintenance of pluripotency.

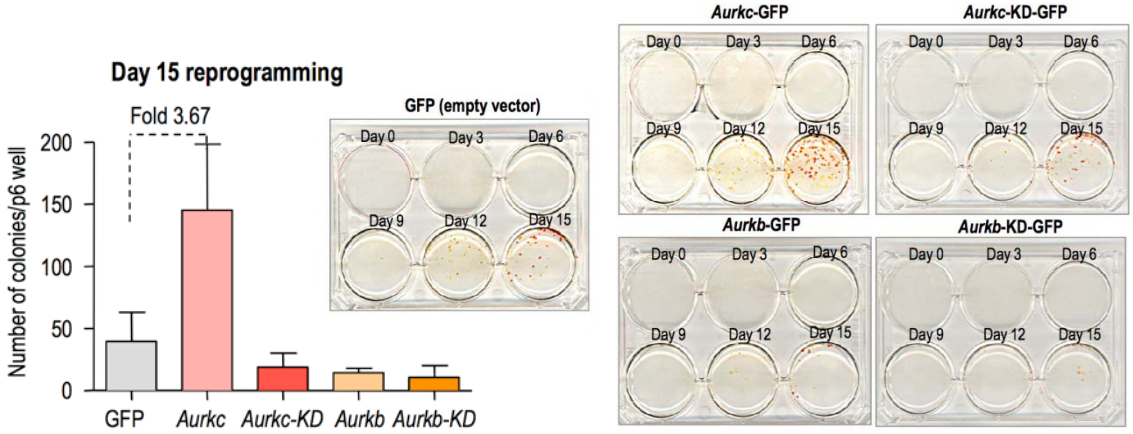


Figure 46. AurkC enhances reprogramming efficiency. Number of Alkaline Phosphatase stained colonies at days 9, 12 and 15. Embryonic fibroblasts were transfected one day before pluripotency induction with plasmids expressing GFP (empty vector, EV) or GFP fusion proteins containing wild-type Aurora B (*AurkB* wt), Aurora C (*AurkC* wt) or kinase dead mutant forms of these proteins (*AurkB* KD and *AurkC* KD). A huge increase in this process is observed upon Aurora C overexpression Graph shows the number of stem positive colonies at day 15 of the reprogramming process. Fold change of Aurora C versus EV is shown. On the right, pictures showing colonies along the reprogramming process from day 0 to day 15. Alkaline Phosphatase staining (red) during reprogramming of Rosa-rtTTA; lenti-4F MEFs.

In order to know whether the effects on reprogramming are due to an increase activation of the cell cycle division, we decided to analyse the effects of Aurora C overexpression in MEFs proliferation. Since no differences were found in terms of cell growth, the observed effect on reprogramming efficiency must be due to a non-mitotic function of Aurora C.

3.3. Active epigenetics marks upon Aurora C overexpression

The reprogramming process is accompanied by several changes in the epigenome mostly on pluripotency-related or developmentally regulated gene promoters. These changes are critical to allow a permissive transcriptional program and an *open* and decondensed chromatin state, characteristic of pluripotent cells (Bernstein *et al*; 2006).

Since we observed that by overexpressing Aurora C during reprogramming the number of colonies increased, we aimed to go further and analyse how the epigenome was changing during reprogramming upon Aurora B/C induction. We therefore analysed the epigenetic modifications in the above mentioned Aurora B/C overexpressed/deficient murine cells during the first 6 days of reprogramming. This is due to the fact that although the transition from a somatic like chromatin structure to an ESC like structure occurs at day 7 or later, chromatin reorganization occurs early preceding nanog expression during reprogramming (day 6 or earlier) (Mattout A *et al*; 2011). We tested the status of acetylation and methylation marks that correlate with transcriptional activity and the marks presenting the highest degree of correlation with pluripotent state are the acetylation of lysine 9 in histone 3 (H3K9Ac) and the trimethylation of lysine 4 in histone 3 (H3K4me3). These epigenetic marks for transcription activation were upregulated in Aurora C overexpressed cells (Figure 47).

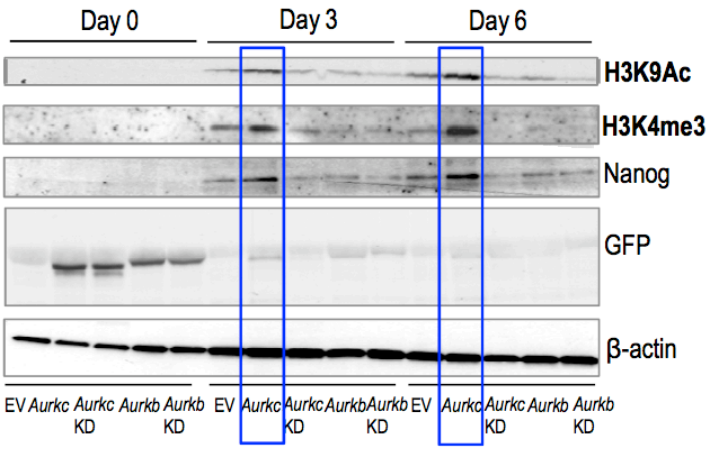
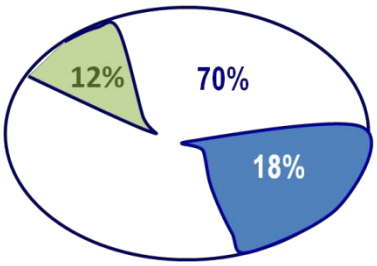


Figure 47. Aurora C epigenetic modifications. Inmunoblot of epigenetic marks at day 0, 3 and 6 of the reprogramming process. Embryonic fibroblasts were transfected one day before pluripotency induction with plasmids expressing GFP (empty vector, EV) or GFP fusion proteins containing wild-type Aurora B (*AurkB* wt), Aurora C (*AurkC* wt) or kinase dead mutant forms of these proteins (*AurkB* KD and *AurkC* KD). The status of H3K9Ac and H3K4me3 histone marks, which correlate with activation of the transcriptional activity was tested.

Overall these data show a differential role of Aurora B and C proteins in pluripotency induction, a question that deserves further investigation in future projects.



Discussion

Discussion

In this work we have investigated the physiological relevance of deregulating the cell cycle kinase Aurora B, which is crucial for a proper segregation of chromosomes during mitosis. Strikingly, we found that its *in vivo* depletion leads to a premature ageing phenotype characterized by an upregulation of apoptosis, aneuploidy and lack of stem cell renewal. This indicates that Aurora B is necessary for tissue regeneration. On the other hand, long-term overexpression of Aurora B results in the development of multiple tumours, accompanied by an increase in aneuploidy and impaired induction of the p53 target $p21^{Cip1}$, phenotype that goes along an altered metabolism. Thus, suggesting that Aurora B overexpression may favor both aneuploidy and $p21^{Cip1}$ down-regulation during tumour development and that Aurora B has functions outside mitosis. Finally, we have found that Aurora C and not Aurora B seems to be required for stemness condition.

1. Aurora B biological function. Consequences of Aurora B deregulation

Aurora B, the catalytic component of the Chromosomal Passenger Complex (CPC), is an essential kinase in the error correction mechanism that ensures proper chromosome segregation when cells divide (Ditchfield et al., 2003; Lampson and Cheeseman, 2011). It has been previously described that Aurora B deregulation (exogenously downregulating/overexpressing the protein) contributes to chromosomal instability and leads to mitotic defects (Hauf et al., 2003) (Terada et al., 1998) (Ota et al., 2002). In this work, we took advantage of genetically modified mouse models for Aurora B depletion/overexpression which allowed us to faithfully analyse the physiological consequences of the protein deregulation *in vivo*.

1.1. Effect of Aurora B depletion in adult mice

Aurora B, due to its critical roles in the cell cycle, it is required for proper cellular proliferation in cultured cells (Hauf et al., 2003; Nguyen et al., 2009) (Fant et al., 2010; Tatsuka et al., 1998), and its genetic inactivation results in embryonic lethality after implantation (Fernandez-Miranda et al., 2011; Fernández-Miranda et al., 2011; Ye et al., 2009). Due to the use of Aurora B inhibitors as antitumoural agents (Ditchfield et al., 2003; Harrington et al., 2004; Hauf et al., 2003), we decided to analyse the consequences of its depletion in adult mammals in order to discriminate the specific effects of the inhibition of Aurora B associated with the use of these inhibitors.

Using a conditional knock-out model we show here that Aurora B is critical for cell proliferation in adult mammals, a feature that has been critical for their consideration as a putative cancer target. Lack of cell proliferation together with increased apoptosis made Aurora B depleted mice present decreased survival. Our results suggest that lack of Aurora B results in defective chromosome segregation that leads to the generation of aneuploid cells together with an induction of p53 and its main target gene p21Cip1

(Figures 13 and 14). Interestingly, aneuploid cells are characterized by the induction of DNA damage response as a consequence of induction of replicative stress (Storchová et al., 2006). The cause of the decreased survival was a clear ageing phenotype together with a loss of body weight, similar to loss of function models for other mitotic regulators previously described (Baker et al., 2004; Pérez de Castro et al., 2013).

These data indicate that the observed phenotype upon Aurora B depletion is due to an impaired proliferation caused by mitotic abnormalities and increased apoptosis and p53 response which together with les regenerative capacity of stem cells generates premature-ageing mice (Figure 15). The upregulation of p53 we observed could be due to an increased stability of the protein. In addition, we have observed an intriguing reduction of stem cell renewal (Figure 18), an observation that could be used to induce tumour arrest by inhibiting the protein in some specific tissue areas.

Two cellular mechanisms can contribute to chemo/radioresistance: inhibition of apoptotic cell death pathways and induction of autophagy, a cell survival response. The phenotype observed in the Aurora B depleted mice was similar to *Aurka*$^{\Delta/\Delta}$ mice (Pérez de Castro et al., 2013). Both models display a premature ageing phenotype, together with less regenerative capacity of tissues and an increased p53 response. However, unlike the senescence induced in *Aurka*$^{\Delta/\Delta}$ mice, Aurora B depleted mice presented increased apoptotic cells which make the inhibition of Aurora B an important tool for the clinic. This data has relevance in cancer therapies since the inhibition of Aurora B could sensitize tumours to anticancer agents that are more selective to cancer cells with high levels of p53/p21^{Cip1}. Since autophagy plays a role in chemotherapy and radiotherapy resistance, analyse this pathway in the *Aurkb*$^{\Delta/\Delta}$ mice would be of interest, because if autophagy is inhibited, the resistance mechanisms to Aurora B inhibitors are much less feasible.

1.2. Aurora B overexpression : Comparison to other mouse models of mitotic regulators

Due to the important role of Aurora B in mitosis and its correlation to tumour grade, it is highly important to know the effect of Aurora B in mammals in terms of tumour development. Here, with the aim of studying the relationship between Aurora B upregulation and cancer an Aurora B ovexpression model was generated. This model was generated also to analyse whether chromosomal instability is the mechanism that drives cancer upon Aurora B overexpression. We have taken advantage of a robust system to modulate the expression of endogenous genes in mammals by using the promoter “hijack” strategy previously used to modulate gene expression in chicken DT40 cells (Fant et al., 2010; Samejima et al., 2008). Replacement of endogenous promoter regions by tetracycline-responsive elements results in a robust control of endogenous gene expression both *in vitro* and *in vivo* eliminating the need to express exogenous genes (Figure 20 and 21). In this way, Aurora B is upregulated ubiquitously in the mice by the addition of tetracycline while Aurora B coding region remains intact.

It is known by extensive studies in murine models that reduced expression of mitotic components, as well as its overactivation, is associated with an increase of spontaneous cancer. Bub1 hypomorphic mice develop lymphomas, lung and liver tumours with high incidence (Jeganathan et al., 2007), whereas heterozygous animals for Mad2 develop benign lung tumours with long latencies (Dobles et al., 2000). Likewise, CenpE heterozygous animals evolve benign lung tumours but also splenic lymphomas (Weaver and Cleveland, 2006; Weaver et al., 2007) and heterozygous BubR1 mice are prone to develop colon adenocarcinomas when carcinogenesis is induced (Dai et al., 2004) or in a background of an APC (min) mutation (Rao et al., 2005). Whereas mutations that inactivate mitotic genes are rarely observed in human cancers, overactivation of these genes is a much more frequent event than their loss or partial loss of function. In this way, the mitotic checkpoint components Mad2, Bub1 or the Aurora B substrate Hec1/Ndc80 overexpression are known to drive aneuploidy and to initiate tumourigenesis in inducible murine models (Sotillo et al., 2007) (Diaz-Rodríguez et al., 2008) (Ricke et al., 2011). These overexpression models result in aneuploidies *in vitro* and in a increase tumour incidence *in vivo*. However, other events that contribute to tumour formation such as gain of oncogenes or loss of tumour suppressors can not be discarded.

The mouse model described here confirms the effect of Aurora B overexpression in the generation of misaligned chromosomes and in triggering a SAC-dependent response (Figure 27 and 29) as recently reported in yeast cells when overexpressing Aurora B in combination with INCENP. In that study the overexpression of both proteins generates defects in chromosome segregation produced by the continuous disruption of kinetochore-microtubule attachments. This disruption collapses the mitotic spindle, thus promoting genomic instability (Munoz-Barrera and Monje-Casas, 2014). Aurora B may be involved in the localization of Mad2 and BubR1, proteins that recognize correct chromosome attachment to spindle microtubules since loss of Aurora B lowers the concentration of Mad2 and BubR1 at the kinetochores (Ditchfield et al, 2003). Here, the mitotic defects caused by Aurora B overexpression, mainly lagging and misaligned chromosomes, are accompanied by increased levels of BubR1 at the kinetochores (Figure 26), in line with previous work showing that Aurora B participates in the recruitment of BubR1, but not Mad2 (Ye et al., 2009).

Aurora B is essential for SAC activation in response to taxol, but its requirement for the SAC in the absence of microtubules is still a matter of debate given the partial effects found in multiple assays and the limited efficiency and specificity of chemical inhibitors. So that, our results are consistent with the concept that Aurora B regulates the SAC at least in part by targeting BubR1 to the kinetochores, function that has been previously postulated on entry into mitosis (Ditchfield et al., 2003).

Moreover, Aurora B overexpressing cells display an increase in the number of multiaster (Figure 26), which imply that Aurora B deregulation provokes the formation of multiple poles/MTOCs thus promoting chromosomes misalignments. These alterations produce with time CIN, as previously postulated (Nguyen et al., 2009; Ota et al., 2002; Terada et al., 1998). CIN and SAC hyperactivation

induce an increase in the length of mitosis, consistent with a reduction in the proliferation capacity of cells (Figure 25) as it has been previously shown for other mitotic regulators (Zhang et al., 2004)(Sotillo et al., 2007). The observed effect on SAC upon Aurora B overexpression seems to be similar to the effect found upon its depletion (Girdler et al., 2006) in which an increase in DOM is seen upon Aurora B inhibition. Taken together, our results are consistent with previous models of aneuploidy in which CIN has an *in vitro* negative effect on cellular viability (Sotillo et al., 2007; Torres et al., 2008).

2. Is Aurora B causally associated to tumourigenesis?

Genomic studies have suggested the presence of an expression signature in which the overexpression of about 70 genes is associated with chromosomal instability (CIN) in cancer (Carter et al., 2006). This signature is enriched in regulators of the cell division machinery. The genes implicated in all of these processes are known to be misregulated in tumours and it is now well accepted that both increases and decreases in the expression of mitotic genes can induce CIN. Within all of them, some genes that are involved in the regulation of chromosome segregation such a Aurora B, Mad2 or Bub1, correlate with tumour grade and prognosis in a variety of human tumours (Shigeishi et al., 2001; Tanaka et al., 2008).

An unresolved question in cancer is whether tumour-associated overexpression of proteins is just a consequence of cell transformation or whether it indicates a causal role for the protein. This question is especially difficult to address in the case of cell cycle regulators, given the general increase in cell proliferation rates observed in tumour cells. Defects in the cell cycle machinery are commonly linked to cancer, either by promoting unscheduled proliferation, or by allowing the introduction of genomic abnormalities in the daughter cells. One of the hypotheses is that aneuploidy drives tumourigenesis by a mechanism in which oncogenes are gained or tumour suppressor genes are lost (Lengauer et al., 1998). However, at the moment, the link between aneuploidy induction and tumour development still remains unclear. Aurora B is overexpressed in many tumour types and a correlation between its levels and tumour grade/poor clinical prognosis has been proposed (Chieffi et al., 2004; Gibson et al., 2008; Hegyi et al., 2012; Smith et al., 2005; Sorrentino et al., 2005; Tanaka et al., 2008). Previous cellular studies indicate that sustained overexpression of Aurora B in murine epithelial cells induces tetraploidy and chromosomal instability, increasing the oncogenic potential of cultured cells (Ota et al., 2002) (Fant et al., 2010; Sotillo et al., 2007) but the *in vivo* tumourigenesis potential of Aurora B overexpression by itself has not been proven so far.

Does Aurora B has a causal role in tumourigenesis by aneuploidy induction? Though correlations between Aurora B level of expression and the severity of tumours have been described in humans (Table 1), there is no a genetic study of causal connection between Aurora B, aneuploidy and cancer. Our ability to chronically induce Aurora B overexpression in the mouse allowed us to analyse the consequences of the resulting mitotic defects during ageing due to Aurora B overexpression. Overexpression of Aurora B *in vivo* resulted in a significant increase in aneuploidy accompanied by a dramatic susceptibility to

spontaneous tumours (Figures 34 and 37). Thanks to this study we can now state that Aurora B, which is overexpressed in many human tumours (Table 1) is causally associated with tumour development. The aneuploid levels in the Aurora B overexpressing mice at 4 months of induction was low (3% of cells were aneuploid) as found in the hyperactivated model for Mad2 (5.7% of aneuploidy) but both predisposed to the appearance of a wide range of lethal tumours. However, CENP-E defective mice present 35% of aneuploidy in their blood cells, though this alteration induces only 10% of tumour appearance as in the case of Bub1-overexpressing mice that present 30% of aneuploidy in lymphocytes after 5 months of induction whereas splenic lymphomas only present 12% of aneuploid cells. Interestingly, an excess of CIN is known to act in a tumour suppressive manner (Rao et al.; 2005; Weaver et al.; 2007) because cells are not able to tolerate high levels of aneuploidy and die.

Whereas Aurora B-overexpressing tumours presented 15% of aneuploidy (Figure 38), similar to CENP-E tumoural samples (17% of aneuploid cells), other tumours produced upon overexpression of mitotic regulators such as Hec1 have around 40% of aneuploid cells (Schvartzman et al., 2010), indicating that the tumourigenesis obtain upon Aurora B overexpression was not only due to aneuploidy induction. Most probably, other factors, such as tumour suppressors are implicated as previously observed in other mitotic models (Schvartzman et al., 2011).

In our model, peripheral blood cells as well as splenocytes display higher frequency of cells with less than a diploid complement of chromosomes than those with more than 2n. There was a bias towards chromosome loss although chromosome gains were also frequently observed. This fact may occur because cells that have acquired a rare transformative karyotype through multiple chromosome missegregations are likely to lose that karyotype in the next round of cell division.

Recently, it has been hypothesized that Aurora B is a critical target through which overexpressed Bub1 drives aneuploidization and tumourigenesis (Ricke et al., 2011). There are striking similarities between Aurora B and Bub1 overexpression models. Lagging and chromosome misalignments that are seen upon Aurora B overexpression result in aneuploidy as seen in Bub1 overexpressing cells. Nevertheless, no differences in the DOM neither on mitotic checkpoint signaling were observed upon Bub1 overexpression. Moreover, Bub1 overexpression in MEFs leads to premature sister chromatid separation (PMCS) and cells are able to maintain an arrest in response to nocodazole or taxol, similar to wild type cells. These differences with Aurora B overexpression model may be related to the levels of mitotic checkpoint hyperactivation or to separate effects of both proteins on regulating the checkpoint. Regarding the tumour spectrum found in Bub1 model, this was similar to the Aurora B overexpression model: lymphomas, sarcomas and lipogranulomas were mainly detected in Aurora B and Bub1 overexpression models, although the tumour incidence was higher in the Aurora B overexpression model (93%) compared to Bub1 overexpressing mice (62-71%). In both models, the resulting tumours present similar aneuploid levels (12% and 15% in Bub1 and Aurora B splenic lymphomas). Our data are consistent with the idea of Ricke RM and colleagues that the tumourigenesis and aneuploidization they found could

be due to Aurora B hyperactivation (Ricke et al., 2011). Indeed, we have demonstrated that increased rates of aneuploidy can enhance spontaneous tumourigenesis during ageing, although tumourigenesis was a late event that did not occur in all tissues.

3. Aurora B role in modulating p53 activity

We were initially surprised by the fact that in Aurora B overexpressing mice, spleen tumours were much more frequent than epithelial tumours, such as lung or liver cancers commonly found in other CIN models (Kumari et al., 2014; Schvartzman et al., 2011) and the levels of aneuploidy found in our tumours were not as higher as in other mitotic models (Schvartzman et al., 2011; Ricke et al., 2011), which led us think that another mechanism was taking place.

We reported previously that p53 is induced in Aurora B null embryos (Fernández-Miranda et al., 2011) in agreement with the proposal that Aurora B-dependent phosphorylation of p53 may result in the increased degradation of this transcription factor (Gully et al., 2012). Inactivation of Aurora B also results in increased p53 activity and the subsequent induction of the cell cycle inhibitor $p21^{Cip1}$ in different cell types (Gully et al., 2012; Wu et al., 2011). Indeed, our group has recently described that inactivation of Aurora B leads to increased transcription of $p21^{Cip1}$(Trakala et al., 2013).

It is known that p53 maintains genomic stability in mice largely by p21 induction (Barboza et al., 2006). Indeed, Aurora B deficiency leads to an enhanced expression of $p21^{Cip1}$ resulting in aberrant Cdk activity and cell cycle progression (Lens et al., 2010; Trakala et al., 2013). Furthermore, it is known that p53 deficiency leads to cell cycle deregulation and aneuploidy (Negrini et al., 2010) and accelerates CIN and tumour formation in different mouse models by facilitating the propagation of genetically defective cells as a result of checkpoint loss. In addition, a correlation between abnormal p53 levels and aneuploidy has been described in human tumours (Thompson and Compton, 2010).

Precisely, pharmacologic inhibition of Aurora B in cancer cells, increased p53 protein level and its target genes thus leading to an inhibition of tumour growth (Li et al., 2013). On the contrary, p53 deficiency *in vitro* leads to cell cycle deregulation and aneuploidy (Negrini et al., 2010). However, the relevance of this interaction *in vivo* has not been tested. Similar networks have been described for Aurora A and p53 (Chiang, 2012; Hsueh et al., 2013; Katayama and Sen, 2011; Wu et al., 2012). All of these data led us to think that the tumourigenesis observed in the Aurora B overexpressing mice may be a consequence of the possible role of Aurora B in modulating p53 activity *in vivo*. Indeed, it has been hypothesized that conversion of diploid progenitor cells into cells that are aneuploid takes two steps. First, reduction in chromosome segregation fidelity occurs and second, acquisition of tolerance for a non-diploid genome that can arise through inactivation of the p53 pathway (Thompson and Compton, 2010).

In this study we found that overexpressed Aurora B induces mitotic defects that lead to aneuploidy and this generates tumour induction and $p21^{Cip1}$ downregulation (Figures 32 and 34). Some studies argued that p53 deficiency alone is insufficient to generate aneuploidy and that only when

combining p53 deficiency with elevated chromosome missegregation rates, p53 generates tolerance for the propagation of aneuploid cells (Thompson and Compton, 2010). Our data fit well with the observation that many tumour cells exhibit both aneuploidy and defects in the p53 pathway (Campomenosi et al., 1998) (Tomasini et al., 2008); (Schvartzman et al., 2011), however, we do not know whether this permissive background is a pre-requisite for the development of aneuploidy or it is developed as an adaptation to overcome it. Likely, these two factors may contribute to tumour formation in specific cell types (Duesberg et al., 1998) (Duesberg et al., 1999) (Holland and Cleveland, 2009). Moreover, mutations in tumour suppressor genes tend to occur in the late stages of tumour formation, and such tumours are in the majority of cases aneuploid (Auer et al., 1994) (Baker et al., 1990). Most probably, loss of $p21^{Cip1}$ eliminates the checkpoints that arrest polyploidy and aneuploid cells generated upon Aurora B overexpression so that the resulting cells are prone to become transformed.

Furthermore, we observed that Aurora B overexpressed-tumours presented a downregulation of p21 and p53 levels which suggests that Aurora B is indeed a critical regulator of p53 *in vivo* and that overexpression of this kinase in cancer may lead to deficient p53 function and reduced levels of p53 targets such as the cell cycle inhibitor $p21^{Cip1}$. These results are also in agreement with an inverse correlation between Aurora B and $p21^{Cip1}$ mRNA levels in human B-ALL (Fig. 41). It is likely that aneuploidy and defective p53 expression contribute to tumour formation as a result of Aurora B hyperactivation (Figure 48).

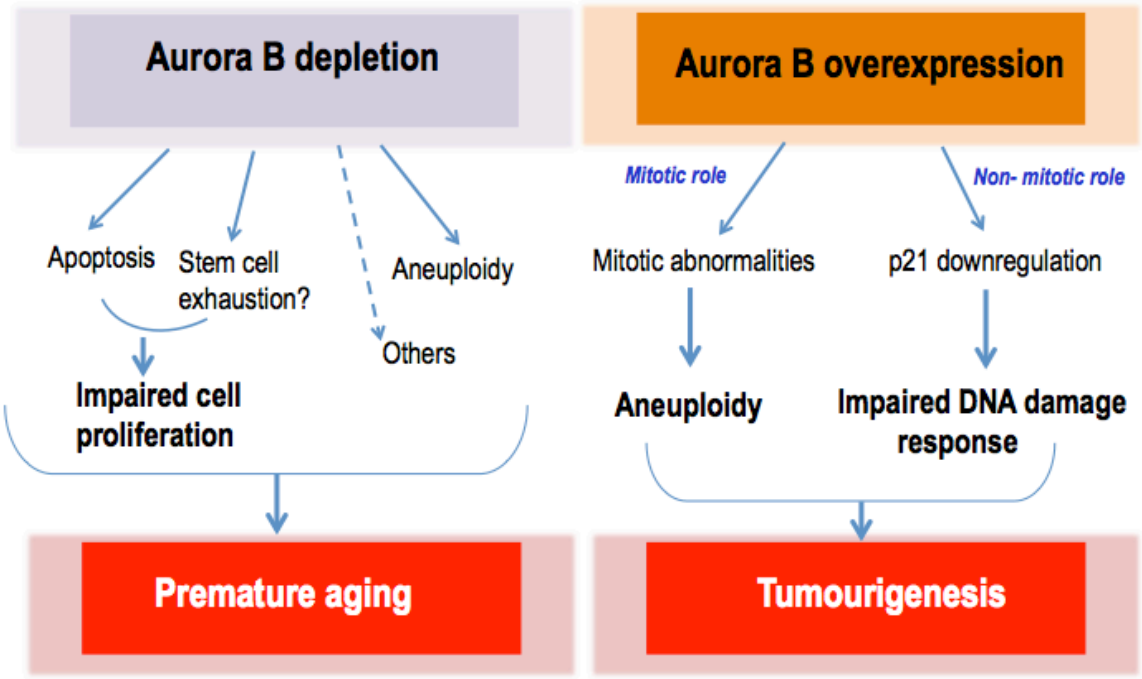


Figure 48. Global scheme of the the outcomes obtained from Aurora B depletion and overexpression models. Aurora B depletion *in vivo* leads to a premature ageing phenotype as a consequence of impaired cell proliferation and aneuploidy. On the other hand, the overexpression of Aurora B induces mainly aneuploidy and a defective DNA damage response and as a consequence, tumourigenesis is induced.

4. Aurora B and metabolism

Aurora kinases are widely known due to its role in mitosis. However, do Aurora kinases have a role outside mitosis? While the main focus on Aurora-A has been its actions in mitosis or cancer, it is increasingly apparent that the protein has important functions in non-mitotic cells, generally in cases in which the protein is overexpressed. Indeed, it has been reported its role in microtubule dynamics, cell migration, and polarity (neurite extension) (Lorenzo et al., 2009) (Yamada et al., 2010) (Mori et al., 2009), induction of disassembly of cilia (Pugacheva et al., 2007), and regulation of intracellular calcium signaling (Plotnikova et al., 2011). Aurora B is a major histone H3 kinase that mediates gene silencing epigenetically during cell differentiation, independently of the cell cycle (Sabbattini et al., 2007). Aurora B exerts this function by deposition of the double epigenetic modification H3K9me3/S10ph at silent genes in differentiated mesenchymal stem cells which promotes HP1 β displacement from chromatin (Sabbattini et al., 2014). Furthermore, several evidences have recently suggested a role for Aurora B in G1-S transition as revealed by acute elimination of the protein in G0 cells probably by regulating the mTOR pathway and/or the levels of the p53 effector $p21^{Cip1}$ (Suzuki et al., 2000; Trakala et al., 2013). Moreover, some years ago a study of Tárdaguila and collaborators described a role for Aurora B in the regulation of transcription initiation of thyroid receptor (T3)- responsive genes (Tardáguila et al., 2011). All these results indicated that Aurora B could have a role outside the mitotic field.

The surprising metabolic phenotype observed in Aurora B overexpressing mice led us to think that Aurora B has non-mitotic functions by regulating different targets. Ample evidence has shown that the metabolic alterations are critical for the growth, proliferation and survival of tumour cells. Our results suggests that there is a tendency in the Aurora B overexpressing mice to consume less energy and to present less metabolic flexibility (due to a higher and flatter RQ value than wild type mice). Both parameters explain our macroscopic observations in terms of higher weight and steatosis and also indicates that Aurora B overexpressing mice consume less fat and more glucose than wild-type mice, in concordance with the fat storage found in several tissues. Moreover, transgenic mice eat slightly more and their activity is a little higher than in wild type. Since after 1.5 months of Aurora B overexpression we can see tendencies but not significative differences, the analysis of the Aurora B overexpressing mice at later doxycycline period would be crucial in order to decipher the metabolic outcome. These are preliminary results that will be further completed by analyzing whether a food intake defect exits in the transgenic mice versus the control mice and if so, we could study possible hypothalamus defects testing the main hypothalamic peptides. It would be of interest to check the levels of metabolites in serum and to analyse the levels of Uncoupling protein 1 (UCP1) in BAT of wild-type and inducible Aurora B mice and also to analyse in these animals the possible transcriptional differences in fat or thyroid.

5. Aurora C and pluripotency

While Aurora A and B are widely expressed in dividing cells, Aurora C pattern is restricted to spermatogenesis and oocyte fertilization. Aurora C deficiency results in viable mice with subfertility defects such as heterogeneous chromatin condensation, loose acrosomes and blunted sperm heads (Kimmins et al., 2007a). Interestingly, Aurora C displays frameshift mutations in infertile patients with abnormal spermatozoa characterized by large heads and increased chromosomal content (Dieterich et al., 2007a), suggesting a critical role in mammalian spermatogenesis. Additional loss-of-function studies of Aurora C in oocytes suggest a critical role for this protein in meiosis (Sharif et al., 2010; Yang et al., 2010). It is important to note that Aurora C is highly expressed in oocytes and during the first embryo divisions to reach a minimum expression during the blastocyst stage (Hamatani et al., 2004). There is no Aurora C expression in blastocysts, implanted embryos or 3T3 fibroblasts. Why germ cells express Aurora C is unknown. On the other hand, it has been hypothesized that this protein may act as a CPC component (Sasai et al., 2004; Slattery et al., 2008; Yan et al., 2005). However, its relevance in CPC function and mitosis has not been fully elucidated given the reduced endogenous expression levels in cultured cell lines.

Since Aurora C, the unknown member of the family, is very close to Aurora B one would expect that the overexpression of Aurora C could have the same consequences as the overexpression of Aurora B, however this is not the case in terms of stemness induction. Based on sequence and functional analyses Aurora B and C are very similar, however why germ cells express Aurora C is unclear. Could they have different regulatory functions? Previous data from our laboratory indicate that Aurora B null pre-implantation embryos developed normally and were positively stained for phospho-histone 3, what suggested that Aurora B could be dispensable due to the presence of Aurora C. Aurora C was demonstrated to be responsible of these early cell divisions; whereas once the blastocyst is formed this kinase is replaced by Aurora B (except germ cells; Fernández-Miranda et al., 2011) (Figure 44). These data were confirmed by a study in which it was observed that the posttranscriptional regulation of *Aurkc* mRNA and the greater stability of the protein ensured enough kinase activity, despite loss of Aurora B, to support early embryonic cell divisions (Schindler et al., 2012). Moreover, Sabbattini P. and collaborators (Sabbattini et al., 2007) have previously identified a new role for Aurora B in epigenetically marking silent chromatin during cell differentiation, which made us think of an opposite role of both kinases in stemness function.

In this work, we verified that Aurora C is expressed in ES cells and iPCs. In addition, the ability of Aurora C to increase the efficiency of reprogramming suggests for the first time the relevance of this protein in pluripotency induction. Pluripotency requires activation of specific gene expression programs and long-term silencing of genes that specify cell fates. Epigenetic modifications of the core histones form complex combinations on nucleosomes that are believed to reinforce activating and silencing effects of

transcription factors and participate in the maintenance of cellular memory of transcription states (Jenuwein and Allis, 2001; Turner, 2002). Since we observed an upregulation of epigenetic marks only when overexpressing Aurora C, we strongly believe that one of the different aspects between these two proteins is their comparative ability to induce epigenetic changes during reprogramming. So that the role of Aurora C is because of its intrinsic function and not because of their differential pattern of expression to that of Aurora B. Aurora C possibly has a new role in stemness maintaining by acting as a regulator of the epigenetic status of undifferentiated cells.

These preliminary data supporting the role of Aurora C in pluripotency, along the relevance of Aurora C during meiosis (Dieterich et al., 2007a; Sharif et al., 2010; Yang et al., 2010) and in early cell divisions indicate that Aurora C display critical roles in germ cells and during early embryo development. We are planning to investigate how Aurora C mediates these changes by checking Aurora C promoter and by performing a genome-wide analysis of the putative binding of Aurora B and Aurora C to DNA in order to find the *in vivo* binding sites of both kinases to chromatin and to identify functional elements of the genome.

6. Concluding remarks: Therapeutic implication of the use of Aurora B/C inhibitors

The three members of the Aurora kinase family are overexpressed in several types of cancer (Giet et al., 2005; Sorrentino et al., 2005; Tanaka et al., 1999; Zekri et al., 2012; Zhou et al., 1998). Since this discovery, Aurora kinases are considered as important cancer targets (Domenech and Malumbres, 2013; Lens et al., 2010) (Keen and Taylor, 2004) (Pérez de Castro et al., 2008). Indeed, several Aurora inhibitors have been developed during the last decade and most pre-clinical studies showed the potent antiproliferative defects of these compounds, such as Danusertib or AT9283 (Malumbres and Perez de Castro, 2014). Whereas initial efforts were focused on Aurora A, recent data suggest that Aurora B is a relevant therapeutic target for cancer treatment (Girdler et al., 2006; Girdler et al., 2008). Aurora B inhibitors are antitumoural agents that are at the moment in clinical trials I, II and III, among them, BI811283, AZD1152, GSK1070916 (Malumbres and Perez de Castro, 2014). Because many of the inhibitors can act in a similar manner against the three Aurora members and also against another kinases, it is important to discriminate the specific effects of the inhibition of Aurora B associated with the use of these inhibitors.

Moreover, several phase I and II trials have been initiated to test the effect of Aurora inhibitors in combination with other drugs already approved for cancer treatment. Given the essential role of Aurora B in mitosis, its inhibition could in principle impair the proliferation of any tumour. This makes Aurora B inhibitors potential universal drugs for the treatment of oncologic disorders. The use of Aurora B inhibitors have shown promising results during the last years, however, their inclusion in routine anti-tumour therapies is still far to be achieved. Additional discoveries on the function of Aurora B need to be addressed to improve the efficacy of Aurora B inhibition for the treatment of cancer diseases.

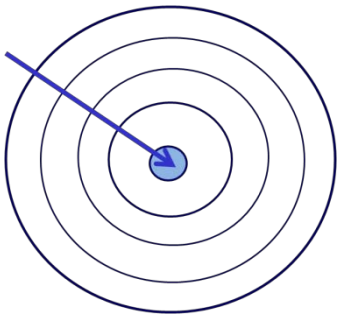
Our findings indicate that Aurora B overexpression can lead *per se* to cancer development. This phenotype is likely produced by a combination of chromosomal instability induction and $p21^{Cip1}$ down-regulation during tumour development. Some Aurora B inhibitors such as Barasertib or AZD1152 induce apoptosis and are at the moment in clinical trials I/II (Yamauchi et al., 2013) (Baldini et al., 2013; Malumbres and Pérez de Castro, 2014), which is in concordance with the observed induction of Caspase-3 positive cells in Aurora B depleted mice. One of the most interesting uses of Aurora B inhibitors is their ability to potentiate the responses induce by other agents. The newly discovered roles of Aurora B in aneuploidy and differentiation (this work), offer new possibilities to explore further combinations. DNA repair inhibitors or agents that selectively kill aneuploid cells can be interesting candidates for combination therapies. Different screenings must be considered to increase the differentiation and apoptotic phenotype induced by these inhibitors.

The cellular stress that comes from chromosome imbalances, make aneuploid cells more sensitive to specific compounds, which can be used as a cancer strategy (Torres et al., 2008). Finding ways to inhibit it or even hyper-induce it, could have significant implications for anticancer therapies. Furthermore, treatment of cells with Aurora B inhibitors induces p53-dependent apoptosis in human leukaemia cells (Ikezoe et al., 2010), representing a major opportunity for anti-cancer drugs.

Different markers have been used to monitor the efficacy of Aurora inhibitors, including mitotic index, spindle bipolarity and chromosome alignment (Chakravarty, 2011), nuclear volume (Perez de Castro, 2013) or the percentage of aligned spindles (Palani, 2013). High proliferative rates have also been associated with tumours that better respond to Aurora B inhibitors, since proliferation requires Aurora B activity. In this sense, Aurora B could be used as a biomarker to test the proliferation status of tumours (Mendiola et al., 2009). The crosstalk between Aurora B and p53 (Wu et al., 2011) makes this protein a potential marker for a more potent effect of Aurora B inhibitors. As Aurora B inhibits p53 (Gully et al., 2012), it can be postulated that a funcitonal p53 pathway could be reactivated upon Aurora B inhibition and therefore sensitize cells to this treament. p53 could be studied to confirm its utility as a biomarker for Aurora B inhibitors efficacy. Furthermore, genomic sequencing, transcriptomics or the analysis of the metabolome should be used to identify new biomarkers.

This finding adds to a wide number of studies showing that deregulation of a single gene implicated in the mitotic machinery is sufficient to contribute to tumour initiation. Understanding the role of such an important mitotic kinase as Aurora B is crucial for deciphering disease pathogenesis and may also lead to new avenues for treating human cancers.As a whole, this study sheds light on the mechanisms by which Aurora B gain of expression induces *in vivo* tumourigenesis and chromosomal instability whereas its complete depletion impairs cell proliferation, a feature that has been critical for their consideration as putative cancer target.

Our data is of special relevance in tumourigenesis because it implies that Aurora B inhibition could sensitize tumours to anticancer compounds that work better against cancer cells with a p53 null background. Aurora inhibitors could possibly have an effect in restoring p53 activity at least in some specific tumours, so that it could be used as a prognostic biomarker for tumour progression and patient survival.



Conclusions/ Conclusiones

Conclusions

1. Depletion of Aurora B *in vivo* impairs cell proliferation due to the generation of polyploid/aneuploid cells accompanied by an induction of the cell cycle regulator p53 and its main target $p21^{Cip1}$. This results in defective tissue regeneration that leads to a premature ageing phenotype.

2. Overexpression of Aurora B *in vitro* does not affect the other CPC components but induces increased loading of the mitotic checkpoint protein BubR1, chromosome segregation defects, and aneuploidy.

3. Long-term overexpression of Aurora B impairs the DNA damage response and represses the cell cycle inhibitor $p21^{Cip1}$ *in vitro* and *in vivo*.

4. Aurora B overexpressing mice are tumor prone, they mainly present follicular B cell type lymphomas and they present higher aneuploid levels than control mice.

5. Overexpression of Aurora B causes metabolic defects in adult mammals, mainly white adipose tissue (WAT) inside the brown adipose tissue (BAT), liver steatosis and adipose hyperplasia.

6. Aurora C overexpression enhances the number of stem cells colonies during the reprogramming process and upregulates several active epigenetic marks, suggesting a specific role for this kinase in pluripotent cells.

Conclusiones

1. La ausencia de Aurora B *in vivo* impide una correcta proliferación celular, produciendóse como consecuencia poliploidías/aneuploidías acompañadas de una inducción del regulador del ciclo celular p53 y su principal diana, $p21^{Cip1}$. Como consecuencia se produce una regeneración deficiente de los tejidos que da lugar un fenotipo de envejecimiento prematuro.

2. La sobre-expresión de Aurora B *in vitro* no afecta a otros components del CPC pero induce un aumento en los niveles de la proteína de control mitótico BubR1, defectos en la segregación cromosómica y aneuploidía.

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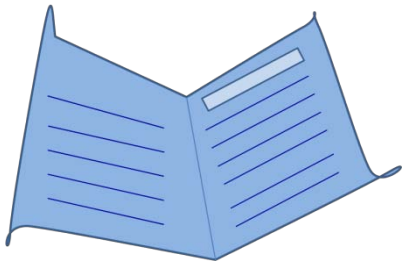
3. La sobre-expresión a largo plazo de Aurora B disminuye la respuesta al daño al DNA y reduce la expresión del inhibidor del ciclo celular $p21^{Cip1}$ *in vitro* e *in vivo*.

4. Los ratones que sobre-expresan Aurora B presentan una mayor incidencia de tumores, principalmente linfomas de tipo folicular los cuales se caracterizan por unos niveles altos de aneuploidía.

5. La sobre-expresión de Aurora B produce defectos metabólicos en ratones adultos, principalmente presencia de tejido adiposo blanco en el tejido adiposo marrón, esteatosis en hígado e hiperplasia de adipocitos.

6. La sobre-expresión de Aurora C provoca un incremento en el número de colonias pluripotentes durante la reprogramación celular y una potenciación en marcas epigenéticas de activación de cromatina, sugiriendo que Aurora C puede tener un papel especifico en la formación de células pluripotentes.



References

References

Abad, M., Mosteiro, L., Pantoja, C., Cañamero, M., Rayon, T., Ors, I., Graña, O., Megías, D., Domínguez, O., Martínez, D*., et al.* (2013). Reprogramming in vivo produces teratomas and iPS cells with totipotency features. Nature *502*, 340-345.

Adams, R. R., Maiato, H., Earnshaw, W. C., and Carmena, M. (2001). Essential roles of Drosophila inner centromere protein (INCENP) and aurora B in histone H3 phosphorylation, metaphase chromosome alignment, kinetochore disjunction, and chromosome segregation. J Cell Biol *153*, 865-880.

Albertson, D. G., Collins, C., McCormick, F., and Gray, J. W. (2003). Chromosome aberrations in solid tumors. Nat Genet *34*, 369-376.

Andrews P. D.,Ovechkina Y, Morrice N., Wagenbach M., Duncan K., Worderman L.,Swedlow J.R. (2004) Aurora B regulates MCAK at the mitotic centromere. Dev Cell *6*, 253-268.

Araki, K., Nozaki, K., Ueba, T., Tatsuka, M., and Hashimoto, N. (2004). High expression of Aurora-B/Aurora and Ipll-like midbody-associated protein (AIM-1) in astrocytomas. J Neurooncol *67*, 53-64.

Auer, G. U., Heselmeyer, K. M., Steinbeck, R. G., Munck-Wikland, E., and Zetterberg, A. D. (1994). The relationship between aneuploidy and p53 overexpression during genesis of colorectal adenocarcinoma. Virchows Arch *424*, 343-347.

Baker, D. J., Jeganathan, K. B., Cameron, J. D., Thompson, M., Juneja, S., Kopecka, A., Kumar, R., Jenkins, R. B., de Groen, P. C., Roche, P., and van Deursen, J. M. (2004). BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice. Nat Genet *36*, 744-749.

Baker, S. J., Preisinger, A. C., Jessup, J. M., Paraskeva, C., Markowitz, S., Willson, J. K., Hamilton, S., and Vogelstein, B. (1990). p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Res *50*, 7717-7722.

Bakhoum, S. F., Genovese, G., and Compton, D. A. (2009a). Deviant kinetochore microtubule dynamics underlie chromosomal instability. Curr Biol *19*, 1937-1942.

Bakhoum, S. F., Thompson, S. L., Manning, A. L., and Compton, D. A. (2009b). Genome stability is ensured by temporal control of kinetochore-microtubule dynamics. Nat Cell Biol *11*, 27-35.

Baldini, E., Tuccilli, C., Prinzi, N., Sorrenti, S., Antonelli, A., Gnessi, L., Catania, A., Moretti, C., Mocini, R., Carbotta, G*., et al.* (2013). The dual Aurora kinase inhibitor ZM447439 prevents anaplastic thyroid cancer cell growth and tumorigenicity. J Biol Regul Homeost Agents *27*, 705-715.

Barboza, J. A., Liu, G., Ju, Z., El-Naggar, A. K., and Lozano, G. (2006). p21 delays tumor onset by preservation of chromosomal stability. Proc Natl Acad Sci U S A *103*, 19842-19847.

Barr, A. R., and Gergely, F. (2007). Aurora-A: the maker and breaker of spindle poles. J Cell Sci *120*, 2987-2996.

Barr, F. A., Silljé, H. H., and Nigg, E. A. (2004). Polo-like kinases and the orchestration of cell division. Nat Rev Mol Cell Biol *5*, 429-440.

Bartek, J., Lukas, C., and Lukas, J. (2004). Checking on DNA damage in S phase. Nat Rev Mol Cell Biol *5*, 792-804.

Bayliss, R., Sardon, T., Vernos, I., and Conti, E. (2003). Structural basis of Aurora-A activation by TPX2 at the mitotic spindle. Mol Cell *12*, 851-862.

Beard, C., Hochedlinger, K., Plath, K., Wutz, A., and Jaenisch, R. (2006). Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. Genesis *44*, 23-28.

Biggins, S., and Murray, A. W. (2001). The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. Genes Dev *15*, 3118-3129.

Bischoff, J. R., Anderson, L., Zhu, Y., Mossie, K., Ng, L., Souza, B., Schryver, B., Flanagan, P., Clairvoyant, F., Ginther, C.*, et al.* (1998). A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. EMBO J *17*, 3052-3065.

Bishop, J. D., and Schumacher, J. M. (2002). Phosphorylation of the carboxyl terminus of inner centromere protein (INCENP) by the Aurora B Kinase stimulates Aurora B kinase activity. J Biol Chem *277*, 27577-27580.

Bolton, M. A., Lan, W., Powers, S. E., McCleland, M. L., Kuang, J., and Stukenberg, P. T. (2002). Aurora B kinase exists in a complex with survivin and INCENP and its kinase activity is stimulated by survivin binding and phosphorylation. Mol Biol Cell *13*, 3064-3077.

Boveri, T. (1902) Über mehrpolige mitosen als mittel zur analyse des zellkerns. Verh. Phys. Med. Ges. Würzburg *35,* 67–90 (in German).

Braun, K. M., Niemann, C., Jensen, U. B., Sundberg, J. P., Silva-Vargas, V., and Watt, F. M. (2003). Manipulation of stem cell proliferation and lineage commitment: visualisation of label-retaining cells in wholemounts of mouse epidermis. Development *130*, 5241-5255.

Brown, J. R., Koretke, K. K., Birkeland, M. L., Sanseau, P., and Patrick, D. R. (2004). Evolutionary relationships of Aurora kinases: implications for model organism studies and the development of anti-cancer drugs. BMC Evol Biol *4*, 39.

Cahill, D. P., Lengauer, C., Yu, J., Riggins, G. J., Willson, J. K., Markowitz, S. D., Kinzler, K. W., and Vogelstein, B. (1998). Mutations of mitotic checkpoint genes in human cancers. Nature *392*, 300-303.

Campomenosi, P., Assereto, P., Bogliolo, M., Fronza, G., Abbondandolo, A., Capasso, A., Bellomo, P. F., Monaco, R., Rapallo, A., Sciutto, A.*, et al.* (1998). p53 mutations and DNA ploidy in colorectal adenocarcinomas. Anal Cell Pathol *17*, 1-12.

Carmena, M., and Earnshaw, W. C. (2003). The cellular geography of aurora kinases. Nat Rev Mol Cell Biol *4*, 842-854.

Carmena, M., Ruchaud, S., and Earnshaw, W. C. (2009). Making the Auroras glow: regulation of Aurora A and B kinase function by interacting proteins. Curr Opin Cell Biol *21*, 796-805.

Carter, S. L., Eklund, A. C., Kohane, I. S., Harris, L. N., and Szallasi, Z. (2006). A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. Nat Genet *38*, 1043-1048.

Castro, A., Arlot-Bonnemains, Y., Vigneron, S., Labbé, J. C., Prigent, C., and Lorca, T. (2002). APC/Fizzy-Related targets Aurora-A kinase for proteolysis. EMBO Rep *3*, 457-462.

Chan, C. S., and Botstein, D. (1993). Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast. Genetics *135*, 677-691.

Cheeseman, I. M., Anderson, S., Jwa, M., Green, E. M., Kang, J., Yates, J. R., Chan, C. S., Drubin, D. G., and Barnes, G. (2002). Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p. Cell *111*, 163-172.

Cheeseman I. M. and Desai A.(2008) Molecular architecture of the kinetochore-microtubule interface. Nat Rev Mol Cell Biol *9*, 33-46.

Chen J., Yin S., Tahir S.K., Zhang H.,Liu X.,Sarthy A.V., McGonigal T.P., Liu Z., Rosenberg S.H., Ng S.C. (2003). Survivin enhances Aurora B-kinase activity and localizes Aurora B in human cells. J Biol Chem *278*, 486-490.

Chiang, C. M. (2012). p53-Aurora A mitotic feedback loop regulates cell cycle progression and genomic stability. Cell Cycle *11*, 3719-3720.

Chieffi, P., Cozzolino, L., Kisslinger, A., Libertini, S., Staibano, S., Mansueto, G., De Rosa, G., Villacci, A., Vitale, M., Linardopoulos, S.*, et al.* (2006). Aurora B expression directly correlates with prostate cancer malignancy and influence prostate cell proliferation. Prostate *66*, 326-333.

Chieffi, P., Troncone, G., Caleo, A., Libertini, S., Linardopoulos, S., Tramontano, D., and Portella, G. (2004). Aurora B expression in normal testis and seminomas. J Endocrinol *181*, 263-270.

Cimini, D., Howell, B., Maddox, P., Khodjakov, A., Degrassi, F., and Salmon, E. D. (2001). Merotelic kinetochore orientation is a major mechanism of aneuploidy in mitotic mammalian tissue cells. J Cell Biol *153*, 517-527.

Cimini, D., Fiorovaranti D., Salmon E.D.,Degrassi F. (2002) Merotelic kinetochore orientation versus chrosome mono-orientation in the origin of lagging chromosomes in human primary cells. J Cell Sci. *115*,507-515.

Cowley, D. O., Rivera-Pérez, J. A., Schliekelman, M., He, Y. J., Oliver, T. G., Lu, L., O'Quinn, R., Salmon, E. D., Magnuson, T., and Van Dyke, T. (2009). Aurora-A kinase is essential for bipolar spindle formation and early development. Mol Cell Biol *29*, 1059-1071.

Dai, W., Wang, Q., Liu, T., Swamy, M., Fang, Y., Xie, S., Mahmood, R., Yang, Y. M., Xu, M., and Rao, C. V. (2004). Slippage of mitotic arrest and enhanced tumor development in mice with BubR1 haploinsufficiency. Cancer Res *64*, 440-445.

de Cárcer, G., Escobar, B., Higuero, A. M., García, L., Ansón, A., Pérez, G., Mollejo, M., Manning, G., Meléndez, B., Abad-Rodríguez, J., and Malumbres, M. (2011). Plk5, a polo box domain-only protein with specific roles in neuron differentiation and glioblastoma suppression. Mol Cell Biol *31*, 1225-1239.

de Martino, M., Shariat, S. F., Hofbauer, S. L., Lucca, I., Taus, C., Wiener, H. G., Haitel, A., Susani, M., and Klatte, T. (2014). Aurora A Kinase as a diagnostic urinary marker for urothelial bladder cancer. World J Urol.

Dent S.F., Gelmon K.A., Chi K.N., Jonker D.J., Wainman N., Capier C.A., Chen E.X., Lyons J.F., Seymour L. (2013) NCIC CTG IND.181: phase I study of AT9283 given as a weekly 24 hour infusion in advanced malignancies.Invest New Drugs. *6*,1522-9.

Dhanasekaran, S. M., Barrette, T. R., Ghosh, D., Shah, R., Varambally, S., Kurachi, K., Pienta, K. J., Rubin, M. A., and Chinnaiyan, A. M. (2001). Delineation of prognostic biomarkers in prostate cancer. Nature *412*, 822-826.

Diaz-Rodríguez, E., Sotillo, R., Schvartzman, J. M., and Benezra, R. (2008). Hec1 overexpression hyperactivates the mitotic checkpoint and induces tumor formation in vivo. Proc Natl Acad Sci U S A *105*, 16719-16724.

Dieterich, K., Soto Rifo, R., Faure, A. K., Hennebicq, S., Ben Amar, B., Zahi, M., Perrin, J., Martinez, D., Sele, B., Jouk, P. S.*, et al.* (2007a). Homozygous mutation of AURKC yields large-headed polyploid spermatozoa and causes male infertility. Nat Genet *39*, 661-665.

Dieterich, K., Soto Rifo, R., Faure, A. K., Hennebicq, S., Ben Amar, B., Zahi, M., Perrin, J., Martinez, D., Sèle, B., Jouk, P. S.*, et al.* (2007b). Homozygous mutation of AURKC yields large-headed polyploid spermatozoa and causes male infertility. Nat Genet *39*, 661-665.

Ditchfield, C., Johnson, V. L., Tighe, A., Ellston, R., Haworth, C., Johnson, T., Mortlock, A., Keen, N., and Taylor, S. S. (2003). Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. J Cell Biol *161*, 267-280.

Do, T. V., Xiao, F., Bickel, L. E., Klein-Szanto, A. J., Pathak, H. B., Hua, X., Howe, C., O'Brien, S. W., Maglaty, M., Ecsedy, J. A.*, et al.* (2014). Aurora kinase A mediates epithelial ovarian cancer cell migration and adhesion. Oncogene *33*, 539-549.

Dobles, M., Liberal, V., Scott, M. L., Benezra, R., and Sorger, P. K. (2000). Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. Cell *101*, 635-645.

Domenech, E., and Malumbres, M. (2013). Mitosis-targeting therapies: a troubleshooting guide. Current opinion in pharmacology *13*, 519-528.

Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J., and Beach, D. (1989). Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. Cell *56*, 829-838.

Duesberg, P., Rasnick, D., Li, R., Winters, L., Rausch, C., and Hehlmann, R. (1999). How aneuploidy may cause cancer and genetic instability. Anticancer Res *19*, 4887-4906.

Duesberg, P., Rausch, C., Rasnick, D., and Hehlmann, R. (1998). Genetic instability of cancer cells is proportional to their degree of aneuploidy. Proc Natl Acad Sci U S A *95*, 13692-13697.

Dutertre, S., Hamard-Péron, E., Cremet, J. Y., Thomas, Y., and Prigent, C. (2005). The absence of p53 aggravates polyploidy and centrosome number abnormality induced by Aurora-C overexpression. Cell Cycle *4*, 1783-1787.

Eckerdt, F., Yuan, J., and Strebhardt, K. (2005). Polo-like kinases and oncogenesis. Oncogene *24*, 267-276.

Emanuele, M. J., Lan, W., Jwa, M., Miller, S. A., Chan, C. S., and Stukenberg, P. T. (2008). Aurora B kinase and protein phosphatase 1 have opposing roles in modulating kinetochore assembly. J Cell Biol *181*, 241-254.

Eyers, P. A., Erikson, E., Chen, L. G., and Maller, J. L. (2003). A novel mechanism for activation of the protein kinase Aurora A. Curr Biol *13*, 691-697.

Fang, G. (2002). Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex. Mol Biol Cell *13*, 755-766.

Fant, X., Samejima, K., Carvalho, A., Ogawa, H., Xu, Z., Yue, Z., Earnshaw, W. C., and Ruchaud, S. (2010). Use of DT40 conditional-knockout cell lines to study chromosomal passenger protein function. Biochem Soc Trans *38*, 1655-1659.

Fernandez-Miranda, G., Trakala, M., Martin, J., Escobar, B., Gonzalez, A., Ghyselinck, N. B., Ortega, S., Canamero, M., Perez de Castro, I., and Malumbres, M. (2011). Genetic disruption of aurora B uncovers an essential role for aurora C during early mammalian development. Development *138*, 2661-2672.

Fernández-Miranda, G., Trakala, M., Martín, J., Escobar, B., González, A., Ghyselinck, N. B., Ortega, S., Cañamero, M., Pérez de Castro, I., and Malumbres, M. (2011). Genetic disruption of aurora B uncovers an essential role for aurora C during early mammalian development. Development *138*, 2661-2672.

Floyd, S., Pines, J., and Lindon, C. (2008). APC/C Cdh1 targets aurora kinase to control reorganization of the mitotic spindle at anaphase. Curr Biol *18*, 1649-1658.

Foran J., Ravandi F., Wierda W., Garcia-Manero G., Verstovsek S., Kadia T., Burger J., Yule M., Langford G., Lyons J., Ayrton J., Lock V., Borthakur G., Cortes J., Kantarjian H.(2014) A phase I and

pharmacodynamic study of AT9283, a small-molecule inhibitor of aurora kinases in patients with relapsed/refractory leukemia or myelofibrosis. Clin Lymphoma Myeloma Leuk.*14*,223-30.

Francisco, L., and Chan, C. S. (1994). Regulation of yeast chromosome segregation by Ipl1 protein kinase and type 1 protein phosphatase. Cell Mol Biol Res *40*, 207-213.

Francisco, L., Wang, W., and Chan, C. S. (1994). Type 1 protein phosphatase acts in opposition to IpL1 protein kinase in regulating yeast chromosome segregation. Mol Cell Biol *14*, 4731-4740.

Fry, A. M. (2002). The Nek2 protein kinase: a novel regulator of centrosome structure. Oncogene *21*, 6184-6194.

García-Higuera, I., Manchado, E., Dubus, P., Cañamero, M., Méndez, J., Moreno, S., and Malumbres, M. (2008). Genomic stability and tumour suppression by the APC/C cofactor Cdh1. Nat Cell Biol *10*, 802-811.
Gassmann, R., Carvalho, A., Henzing, A. J., Ruchaud, S., Hudson, D. F., Honda, R., Nigg, E. A., Gerloff, D. L., and Earnshaw, W. C. (2004). Borealin: a novel chromosomal passenger required for stability of the bipolar mitotic spindle. J Cell Biol *166*, 179-191.

Gibson, S. E., Zeng, W. F., Weil, R. J., and Prayson, R. A. (2008). Aurora B kinase expression in ependymal neoplasms. Appl Immunohistochem Mol Morphol *16*, 274-278.

Giet, R., and Glover, D. M. (2001). Drosophila aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis. J Cell Biol *152*, 669-682.

Giet, R., Petretti, C., and Prigent, C. (2005). Aurora kinases, aneuploidy and cancer, a coincidence or a real link? Trends Cell Biol *15*, 241-250.

Giles F.J., Swords R.T., Nagler A., Hochhaus A., Ottmann O.G., Rizzieri D.A., Talpaz M., Clark J., Watson P., Xiao A., Zhao B., Bergstrom D., Le Coutre P.D., Freedman S.J., Cortes J.E.(2013).MK-0457, an Aurora kinase and BCR-ABL inhibitor, is active in patients with BCR-ABL T315I leukemia. Leukemia *27*, 113-117.

Girdler, F., Gascoigne, K. E., Eyers, P. A., Hartmuth, S., Crafter, C., Foote, K. M., Keen, N. J., and Taylor, S. S. (2006). Validating Aurora B as an anti-cancer drug target. J Cell Sci *119*, 3664-3675.

Girdler, F., Sessa, F., Patercoli, S., Villa, F., Musacchio, A., and Taylor, S. (2008). Molecular basis of drug resistance in aurora kinases. Chem Biol *15*, 552-562.

Glotzer, M. (2005). The molecular requirements for cytokinesis. Science *307*, 1735-1739.

Glover, D. M., Leibowitz, M. H., McLean, D. A., and Parry, H. (1995). Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. Cell *81*, 95-105.

Goto, H., Yasui, Y., Kawajiri, A., Nigg, E. A., Terada, Y., Tatsuka, M., Nagata, K., and Inagaki, M. (2003). Aurora-B regulates the cleavage furrow-specific vimentin phosphorylation in the cytokinetic process. J Biol Chem *278*, 8526-8530.

Gregan, J., Polakova, S., Zhang, L., Tolić-Nørrelykke, I. M., and Cimini, D. (2011). Merotelic kinetochore attachment: causes and effects. Trends Cell Biol *21*, 374-381.

Guertin, D. A., Trautmann, S., and McCollum, D. (2002). Cytokinesis in eukaryotes. Microbiol Mol Biol Rev *66*, 155-178.

Gully, C. P., Velazquez-Torres, G., Shin, J. H., Fuentes-Mattei, E., Wang, E., Carlock, C., Chen, J., Rothenberg, D., Adams, H. P., Choi, H. H.*, et al.* (2012). Aurora B kinase phosphorylates and instigates degradation of p53. Proc Natl Acad Sci U S A *109*, E1513-1522.

Guse, A., Mishima, M., and Glotzer, M. (2005). Phosphorylation of ZEN-4/MKLP1 by aurora B regulates completion of cytokinesis. Curr Biol *15*, 778-786.

Hamatani, T., Carter, M. G., Sharov, A. A., and Ko, M. S. (2004). Dynamics of global gene expression changes during mouse preimplantation development. Dev Cell *6*, 117-131.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Harrington, E. A., Bebbington, D., Moore, J., Rasmussen, R. K., Ajose-Adeogun, A. O., Nakayama, T., Graham, J. A., Demur, C., Hercend, T., Diu-Hercend, A.*, et al.* (2004). VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. Nat Med *10*, 262-267.

Harvey, R. C., Mullighan, C. G., Wang, X., Dobbin, K. K., Davidson, G. S., Bedrick, E. J., Chen, I. M., Atlas, S. R., Kang, H., Ar, K.*, et al.* (2010). Identification of novel cluster groups in pediatric high-risk B-precursor acute lymphoblastic leukemia with gene expression profiling: correlation with genome-wide DNA copy number alterations, clinical characteristics, and outcome. Blood *116*, 4874-4884.

Hauf, S., Cole, R. W., LaTerra, S., Zimmer, C., Schnapp, G., Walter, R., Heckel, A., van Meel, J., Rieder, C. L., and Peters, J. M. (2003). The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. J Cell Biol *161*, 281-294.

Hegyi, K., Egervári, K., Sándor, Z., and Méhes, G. (2012). Aurora kinase B expression in breast carcinoma: cell kinetic and genetic aspects. Pathobiology *79*, 314-322.

Hirota, T., Kunitoku, N., Sasayama, T., Marumoto, T., Zhang, D., Nitta, M., Hatakeyama, K., and Saya, H. (2003). Aurora-A and an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells. Cell *114*, 585-598.

Holland, A. J., and Cleveland, D. W. (2009). Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. Nat Rev Mol Cell Biol *10*, 478-487.

Honda, R., Körner, R., and Nigg, E. A. (2003). Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis. Mol Biol Cell *14*, 3325-3341.

Hsueh, K. W., Fu, S. L., Chang, C. B., Chang, Y. L., and Lin, C. H. (2013). A novel Aurora-A-mediated phosphorylation of p53 inhibits its interaction with MDM2. Biochim Biophys Acta *1834*, 508-515.

Hu, H. M., Chuang, C. K., Lee, M. J., Tseng, T. C., and Tang, T. K. (2000). Genomic organization, expression, and chromosome localization of a third aurora-related kinase gene, Aie1. DNA Cell Biol *19*, 679-688.

Ikezoe, T., Yang, J., Nishioka, C., and Yokoyama, A. (2010). p53 is critical for the Aurora B kinase inhibitor-mediated apoptosis in acute myelogenous leukemia cells. Int J Hematol *91*, 69-77.

Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T., and Weinberg, R. A. (1994). Tumor spectrum analysis in p53-mutant mice. Curr Biol *4*, 1-7.

Jeganathan, K., Malureanu, L., Baker, D. J., Abraham, S. C., and van Deursen, J. M. (2007). Bub1 mediates cell death in response to chromosome missegregation and acts to suppress spontaneous tumorigenesis. J Cell Biol *179*, 255-267.

Jelluma, N., Brenkman, A. B., van den Broek, N. J., Cruijsen, C. W., van Osch, M. H., Lens, S. M., Medema, R. H., and Kops, G. J. (2008). Mps1 phosphorylates Borealin to control Aurora B activity and chromosome alignment. Cell *132*, 233-246.

Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. Science *293*, 1074-1080.

Jeyaprakash, A. A., Klein, U. R., Lindner, D., Ebert, J., Nigg, E. A., and Conti, E. (2007). Structure of a Survivin-Borealin-INCENP core complex reveals how chromosomal passengers travel together. Cell *131*, 271-285.

Kallio, M. J., McCleland, M. L., Stukenberg, P. T., and Gorbsky, G. J. (2002). Inhibition of aurora B kinase blocks chromosome segregation, overrides the spindle checkpoint, and perturbs microtubule dynamics in mitosis. Curr Biol *12*, 900-905.

Kanda, A., Kawai, H., Suto, S., Kitajima, S., Sato, S., Takata, T., and Tatsuka, M. (2005). Aurora-B/AIM-1 kinase activity is involved in Ras-mediated cell transformation. Oncogene *24*, 7266-7272.

Kang, H., Chen, I. M., Wilson, C. S., Bedrick, E. J., Harvey, R. C., Atlas, S. R., Devidas, M., Mullighan, C. G., Wang, X., Murphy, M.*, et al.* (2010). Gene expression classifiers for relapse-free survival and minimal residual disease improve risk classification and outcome prediction in pediatric B-precursor acute lymphoblastic leukemia. Blood *115*, 1394-1405.

Karaiskou, A., Perez, L. H., Ferby, I., Ozon, R., Jessus, C., and Nebreda, A. R. (2001). Differential regulation of Cdc2 and Cdk2 by RINGO and cyclins. J Biol Chem *276*, 36028-36034.

Kastan, M. B., and Bartek, J. (2004). Cell-cycle checkpoints and cancer. Nature *432*, 316-323.
Katayama, H., Ota, T., Jisaki, F., Ueda, Y., Tanaka, T., Odashima, S., Suzuki, F., Terada, Y., and Tatsuka, M. (1999). Mitotic kinase expression and colorectal cancer progression. J Natl Cancer Inst *91*, 1160-1162.

Katayama, H., and Sen, S. (2011). Functional significance of Aurora kinase A regulatory interactions with p53-ERα complex in human breast cancer cells. Horm Cancer *2*, 117-124.

Keen, N., and Taylor, S. (2004). Aurora-kinase inhibitors as anticancer agents. Nat Rev Cancer *4*, 927-936.

Kelly, A. E., and Funabiki, H. (2009). Correcting aberrant kinetochore microtubule attachments: an Aurora B-centric view. Curr Opin Cell Biol *21*, 51-58.

Kelly, A. E., Sampath, S. C., Maniar, T. A., Woo, E. M., Chait, B. T., and Funabiki, H. (2007). Chromosomal enrichment and activation of the aurora B pathway are coupled to spatially regulate spindle assembly. Dev Cell *12*, 31-43.

Kimmins, S., Crosio, C., Kotaja, N., Hirayama, J., Monaco, L., Hoog, C., van Duin, M., Gossen, J. A., and Sassone-Corsi, P. (2007a). Differential functions of the Aurora-B and Aurora-C kinases in mammalian spermatogenesis. Mol Endocrinol *21*, 726-739.

Kimmins, S., Crosio, C., Kotaja, N., Hirayama, J., Monaco, L., Höög, C., van Duin, M., Gossen, J. A., and Sassone-Corsi, P. (2007b). Differential functions of the Aurora-B and Aurora-C kinases in mammalian spermatogenesis. Mol Endocrinol *21*, 726-739.

Kimura, M., Matsuda, Y., Yoshioka, T., and Okano, Y. (1999). Cell cycle-dependent expression and centrosome localization of a third human aurora/Ipl1-related protein kinase, AIK3. J Biol Chem *274*, 7334-7340.

Kleylein-Sohn, J., Westendorf, J., Le Clech, M., Habedanck, R., Stierhof, Y. D., and Nigg, E. A. (2007). Plk4-induced centriole biogenesis in human cells. Dev Cell *13*, 190-202.

Kline-Smith, S. L., Khodjakov, A., Hergert, P., and Walczak, C. E. (2004). Depletion of centromeric MCAK leads to chromosome congression and segregation defects due to improper kinetochore attachments. Mol Biol Cell *15*, 1146-1159.

Kufer, T. A., Silljé, H. H., Körner, R., Gruss, O. J., Meraldi, P., and Nigg, E. A. (2002). Human TPX2 is required for targeting Aurora-A kinase to the spindle. J Cell Biol *158*, 617-623.

Kumari, G., Ulrich, T., Krause, M., Finkernagel, F., and Gaubatz, S. (2014). Induction of p21CIP1 Protein and Cell Cycle Arrest after Inhibition of Aurora B Kinase Is Attributed to Aneuploidy and Reactive Oxygen Species. J Biol Chem *289*, 16072-16084.

Lampson, M. A., and Cheeseman, I. M. (2011). Sensing centromere tension: Aurora B and the regulation of kinetochore function. Trends Cell Biol *21*, 133-140.

Lampson, M. A., and Kapoor, T. M. (2005). The human mitotic checkpoint protein BubR1 regulates chromosome-spindle attachments. Nat Cell Biol *7*, 93-98.

Lan, W., Zhang, X., Kline-Smith, S. L., Rosasco, S. E., Barrett-Wilt, G. A., Shabanowitz, J., Hunt, D. F., Walczak, C. E., and Stukenberg, P. T. (2004). Aurora B phosphorylates centromeric MCAK and regulates its localization and microtubule depolymerization activity. Curr Biol *14*, 273-286.

Lei, Y., Yan, S., Ming-De, L., Na, L., and Rui-Fa, H. (2011). Prognostic significance of Aurora-A expression in human bladder cancer. Acta Histochem *113*, 514-518.

Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1998). Genetic instabilities in human cancers. Nature *396*, 643-649.

Lens, S. M., Voest, E. E., and Medema, R. H. (2010). Shared and separate functions of polo-like kinases and aurora kinases in cancer. Nat Rev Cancer *10*, 825-841.

Lesage, B., Qian, J., and Bollen, M. (2011). Spindle checkpoint silencing: PP1 tips the balance. Curr Biol *21*, R898-903.

Li, D., Zhu, J., Firozi, P. F., Abbruzzese, J. L., Evans, D. B., Cleary, K., Friess, H., and Sen, S. (2003). Overexpression of oncogenic STK15/BTAK/Aurora A kinase in human pancreatic cancer. Clin Cancer Res *9*, 991-997.

Li, J., Hu, H., Lang, Q., Zhang, H., Huang, Q., Wu, Y., and Yu, L. (2013). A thienopyrimidine derivative induces growth inhibition and apoptosis in human cancer cell lines via inhibiting Aurora B kinase activity. Eur J Med Chem *65*, 151-157.

Li, X., Sakashita, G., Matsuzaki, H., Sugimoto, K., Kimura, K., Hanaoka, F., Taniguchi, H., Furukawa, K., and Urano, T. (2004). Direct association with inner centromere protein (INCENP) activates the novel chromosomal passenger protein, Aurora-C. J Biol Chem *279*, 47201-47211.

Lin, Y. S., Su, L. J., Yu, C. T., Wong, F. H., Yeh, H. H., Chen, S. L., Wu, J. C., Lin, W. J., Shiue, Y. L., Liu, H. S.*, et al.* (2006). Gene expression profiles of the aurora family kinases. Gene Expr *13*, 15-26.

Littlepage, L. E., and Ruderman, J. V. (2002). Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit. Genes Dev *16*, 2274-2285.

Liu, D., Vader, G., Vromans, M. J., Lampson, M. A., and Lens, S. M. (2009). Sensing chromosome bi-orientation by spatial separation of aurora B kinase from kinetochore substrates. Science *323*, 1350-1353.

Lorenzo, C., Liao, Q., Hardwicke, M. A., and Ducommun, B. (2009). Pharmacological inhibition of aurora-A but not aurora-B impairs interphase microtubule dynamics. Cell Cycle *8*, 1733-1737.

Losada, A., Hirano, M., and Hirano, T. (2002). Cohesin release is required for sister chromatid resolution, but not for condensin-mediated compaction, at the onset of mitosis. Genes Dev *16*, 3004-3016.

Lu, L. Y., Wood, J. L., Ye, L., Minter-Dykhouse, K., Saunders, T. L., Yu, X., and Chen, J. (2008). Aurora A is essential for early embryonic development and tumor suppression. J Biol Chem *283*, 31785-31790.

Lukas, J., Bohr, V. A., and Halazonetis, T. D. (2006). Cellular responses to DNA damage: current state of the field and review of the 52nd Benzon Symposium. DNA Repair (Amst) *5*, 591-601.

Luo, J., Solimini, N. L., and Elledge, S. J. (2009). Principles of cancer therapy: oncogene and non-oncogene addiction. Cell *136*, 823-837.

Malumbres, M. (2011). Physiological relevance of cell cycle kinases. Physiol Rev *91*, 973-1007.

Malumbres, M., and Barbacid, M. (2001). To cycle or not to cycle: a critical decision in cancer. Nat Rev Cancer *1*, 222-231.

Malumbres, M., and Barbacid, M. (2005). Mammalian cyclin-dependent kinases. Trends Biochem Sci *30*, 630-641.

Malumbres, M., and Pérez de Castro, I. (2014). Aurora kinase A inhibitors: promising agents in antitumoral therapy. Expert Opin Ther Targets *18*, 1377-1393.

Maresca T.J. and Salmon E.D. (2010). Welcome to a new kind of tension: translating kinetochore mechanics into a wait-anaphase signal. J Cell Sci. *123,* 825-835.

Marxer, M., Ma, H. T., Man, W. Y., and Poon, R. Y. (2014). p53 deficiency enhances mitotic arrest and slippage induced by pharmacological inhibition of Aurora kinases. Oncogene *33*, 3550-3560.

Mendiola M, Barriuso J, Mariño-Enríquez A , Redondo A, Domínguez-Cáceres A, Hernández-Cortés G, Pérez-Fernández E, Sánchez-Navarro I, Vara JA, Suárez A, Espinosa E, González-Barón M, Palacios J, Hardisson D (2009). Aurora kinases as prognostic biomarkers in ovarian carcinoma. Hum. Pathol. *40*, 631-638.

Meulenbeld H.J., Mathijssen R.H., Verweij J., de Wit R., de Jonge M.J. (2012) Danusertib, an aurora kinase inhibitor. Expert Opin Investig Drugs. *21,* 383-93.Review.

Miyoshi, Y., Iwao, K., Egawa, C., and Noguchi, S. (2001). Association of centrosomal kinase STK15/BTAK mRNA expression with chromosomal instability in human breast cancers. Int J Cancer *92*, 370-373.

Mori, D., Yamada, M., Mimori-Kiyosue, Y., Shirai, Y., Suzuki, A., Ohno, S., Saya, H., Wynshaw-Boris, A., and Hirotsune, S. (2009). An essential role of the aPKC-Aurora A-NDEL1 pathway in neurite elongation by modulation of microtubule dynamics. Nat Cell Biol *11*, 1057-1068.

Montembault E., Prigent C.(2005) Aurora kinases:therapeutic potential. Drugs Fut.*1,*29.

Morrow, C. J., Tighe, A., Johnson, V. L., Scott, M. I., Ditchfield, C., and Taylor, S. S. (2005). Bub1 and aurora B cooperate to maintain BubR1-mediated inhibition of APC/CCdc20. J Cell Sci *118*, 3639-3652.

Munoz-Barrera, M., and Monje-Casas, F. (2014). Increased Aurora B activity causes continuous disruption of kinetochore-microtubule attachments and spindle instability. Proc Natl Acad Sci U S A.

Musacchio, A., and Hardwick, K. G. (2002). The spindle checkpoint: structural insights into dynamic signalling. Nat Rev Mol Cell Biol *3*, 731-741.

Negrini, S., Gorgoulis, V. G., and Halazonetis, T. D. (2010). Genomic instability--an evolving hallmark of cancer. Nat Rev Mol Cell Biol *11*, 220-228.

Nezi, L., and Musacchio, A. (2009). Sister chromatid tension and the spindle assembly checkpoint. Curr Opin Cell Biol *21*, 785-795.

Nguyen, H. G., Chinnappan, D., Urano, T., and Ravid, K. (2005). Mechanism of Aurora-B degradation and its dependency on intact KEN and A-boxes: identification of an aneuploidy-promoting property. Mol Cell Biol *25*, 4977-4992.

Nguyen, H. G., Makitalo, M., Yang, D., Chinnappan, D., St Hilaire, C., and Ravid, K. (2009). Deregulated Aurora-B induced tetraploidy promotes tumorigenesis. FASEB J *23*, 2741-2748.

Nigg, E. A. (2001). Mitotic kinases as regulators of cell division and its checkpoints. Nat Rev Mol Cell Biol *2*, 21-32.

Nowak, S. J., and Corces, V. G. (2004). Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation. Trends Genet *20*, 214-220.

O'Connell, M. J., Krien, M. J., and Hunter, T. (2003). Never say never. The NIMA-related protein kinases in mitotic control. Trends Cell Biol *13*, 221-228.

Ohi, R., Sapra, T., Howard, J., and Mitchison, T. J. (2004). Differentiation of cytoplasmic and meiotic spindle assembly MCAK functions by Aurora B-dependent phosphorylation. Mol Biol Cell *15*, 2895-2906.

Ota, T., Suto, S., Katayama, H., Han, Z. B., Suzuki, F., Maeda, M., Tanino, M., Terada, Y., and Tatsuka, M. (2002). Increased mitotic phosphorylation of histone H3 attributable to AIM-1/Aurora-B overexpression contributes to chromosome number instability. Cancer Res *62*, 5168-5177.

Pannone, G., Hindi, S. A., Santoro, A., Sanguedolce, F., Rubini, C., Cincione, R. I., De Maria, S., Tortorella, S., Rocchetti, R., Cagiano, S.*, et al.* (2011). Aurora B expression as a prognostic indicator and possible therapeutic target in oral squamous cell carcinoma. Int J Immunopathol Pharmacol *24*, 79-88.

Pardee, A. B. (1974). A restriction point for control of normal animal cell proliferation. Proc Natl Acad Sci U S A *71*, 1286-1290.

Pereira, G., and Schiebel, E. (2003). Separase regulates INCENP-Aurora B anaphase spindle function through Cdc14. Science *302*, 2120-2124.

Perera, D., Tilston, V., Hopwood, J. A., Barchi, M., Boot-Handford, R. P., and Taylor, S. S. (2007). Bub1 maintains centromeric cohesion by activation of the spindle checkpoint. Dev Cell *13*, 566-579.

Perez de Castro, I., de Carcer, G., and Malumbres, M. (2007). A census of mitotic cancer genes: new insights into tumor cell biology and cancer therapy. Carcinogenesis *28*, 899-912.

Perez de Castro, I., de Carcer, G., Montoya, G., and Malumbres, M. (2008). Emerging cancer therapeutic opportunities by inhibiting mitotic kinases. Curr Opin Pharmacol *8*, 375-383.

Peters, J. M. (2006). The anaphase promoting complex/cyclosome: a machine designed to destroy. Nat Rev Mol Cell Biol *7*, 644-656.

Plotnikova, O. V., Pugacheva, E. N., and Golemis, E. A. (2011). Aurora A kinase activity influences calcium signaling in kidney cells. J Cell Biol *193*, 1021-1032.

Price, D. M., Kanyo, R., Steinberg, N., Chik, C. L., and Ho, A. K. (2009). Nocturnal activation of aurora C in rat pineal gland: its role in the norepinephrine-induced phosphorylation of histone H3 and gene expression. Endocrinology *150*, 2334-2341.

Prigent, C., and Dimitrov, S. (2003). Phosphorylation of serine 10 in histone H3, what for? J Cell Sci *116*, 3677-3685.

Pugacheva, E. N., Jablonski, S. A., Hartman, T. R., Henske, E. P., and Golemis, E. A. (2007). HEF1-dependent Aurora A activation induces disassembly of the primary cilium. Cell *129*, 1351-1363.

Pérez de Castro, I., Aguirre-Portolés, C., Fernández-Miranda, G., Cañamero, M., Cowley, D. O., Van Dyke, T., and Malumbres, M. (2013). Requirements for Aurora-A in tissue regeneration and tumor development in adult mammals. Cancer Res *73*, 6804-6815.

Pérez de Castro, I., de Cárcer, G., and Malumbres, M. (2007). A census of mitotic cancer genes: new insights into tumor cell biology and cancer therapy. Carcinogenesis *28*, 899-912.

Pérez de Castro, I., de Cárcer, G., Montoya, G., and Malumbres, M. (2008). Emerging cancer therapeutic opportunities by inhibiting mitotic kinases. Curr Opin Pharmacol *8*, 375-383.

Qi, G., Kudo, Y., Ando, T., Tsunematsu, T., Shimizu, N., Siriwardena, S. B., Yoshida, M., Keikhaee, M. R., Ogawa, I., and Takata, T. (2010). Nuclear Survivin expression is correlated with malignant behaviors of head and neck cancer together with Aurora-B. Oral Oncol *46*, 263-270.

Qian, J., Lesage, B., Beullens, M., Van Eynde, A., and Bollen, M. (2011). PP1/Repo-man dephosphorylates mitotic histone H3 at T3 and regulates chromosomal aurora B targeting. Curr Biol *21*, 766-773.

Rao, C. V., Yang, Y. M., Swamy, M. V., Liu, T., Fang, Y., Mahmood, R., Jhanwar-Uniyal, M., and Dai, W. (2005). Colonic tumorigenesis in BubR1+/-ApcMin/+ compound mutant mice is linked to premature separation of sister chromatids and enhanced genomic instability. Proc Natl Acad Sci U S A *102*, 4365-4370.

Ricke, R. M., Jeganathan, K. B., and van Deursen, J. M. (2011). Bub1 overexpression induces aneuploidy and tumor formation through Aurora B kinase hyperactivation. J Cell Biol *193*, 1049-1064.

Rodríguez, C. I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A. F., and Dymecki, S. M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat Genet *25*, 139-140.

Ruchaud, S., Carmena, M., and Earnshaw, W. C. (2007). The chromosomal passenger complex: one for all and all for one. Cell *131*, 230-231.

Sabbattini, P., Canzonetta, C., Sjoberg, M., Nikic, S., Georgiou, A., Kemball-Cook, G., Auner, H. W., and Dillon, N. (2007). A novel role for the Aurora B kinase in epigenetic marking of silent chromatin in differentiated postmitotic cells. EMBO J *26*, 4657-4669.

Sabbattini, P., Sjoberg, M., Nikic, S., Frangini, A., Holmqvist, P. H., Kunowska, N., Carroll, T., Brookes, E., Arthur, S. J., Pombo, A., and Dillon, N. (2014). An H3K9/S10 methyl-phospho switch modulates Polycomb and Pol II binding at repressed genes during differentiation. Mol Biol Cell *25*, 904-915.

Sakakura, C., Hagiwara, A., Yasuoka, R., Fujita, Y., Nakanishi, M., Masuda, K., Shimomura, K., Nakamura, Y., Inazawa, J., Abe, T., and Yamagishi, H. (2001). Tumour-amplified kinase BTAK is amplified and overexpressed in gastric cancers with possible involvement in aneuploid formation. Br J Cancer *84*, 824-831.

Salaun, P., Rannou, Y., and Prigent, C. (2008). Cdk1, Plks, Auroras, and Neks: the mitotic bodyguards. Adv Exp Med Biol *617*, 41-56.

Samejima, K., Ogawa, H., Cooke, C. A., Hudson, D. F., Hudson, D., Macisaac, F., Ribeiro, S. A., Vagnarelli, P., Cardinale, S., Kerr, A.*, et al.* (2008). A promoter-hijack strategy for conditional shutdown of multiply spliced essential cell cycle genes. Proc Natl Acad Sci U S A *105*, 2457-2462.

Santaguida S and Musacchio A. (2009).The life and miracles of kinetochores. Embo J. *28*,2511-2531.

Santaguida, S., Vernieri, C., Villa, F., Ciliberto, A., and Musacchio, A. (2011). Evidence that Aurora B is implicated in spindle checkpoint signalling independently of error correction. EMBO J *30*, 1508-1519.

Sasai, K., Katayama, H., Stenoien, D. L., Fujii, S., Honda, R., Kimura, M., Okano, Y., Tatsuka, M., Suzuki, F., Nigg, E. A.*, et al.* (2004). Aurora-C kinase is a novel chromosomal passenger protein that can complement Aurora-B kinase function in mitotic cells. Cell Motil Cytoskeleton *59*, 249-263.

Sasai, K., Parant, J. M., Brandt, M. E., Carter, J., Adams, H. P., Stass, S. A., Killary, A. M., Katayama, H., and Sen, S. (2008). Targeted disruption of Aurora A causes abnormal mitotic spindle assembly, chromosome misalignment and embryonic lethality. Oncogene *27*, 4122-4127.

Schindler, K., Davydenko, O., Fram, B., Lampson, M. A., and Schultz, R. M. (2012). Maternally recruited Aurora C kinase is more stable than Aurora B to support mouse oocyte maturation and early development. Proc Natl Acad Sci U S A *109*, E2215-2222.

Schvartzman, J. M., Duijf, P. H., Sotillo, R., Coker, C., and Benezra, R. (2011). Mad2 is a critical mediator of the chromosome instability observed upon Rb and p53 pathway inhibition. Cancer Cell *19*, 701-714.

Schvartzman, J. M., Sotillo, R., and Benezra, R. (2010). Mitotic chromosomal instability and cancer: mouse modelling of the human disease. Nat Rev Cancer *10*, 102-115.

Sen, S., Zhou, H., and White, R. A. (1997). A putative serine/threonine kinase encoding gene BTAK on chromosome 20q13 is amplified and overexpressed in human breast cancer cell lines. Oncogene *14*, 2195-2200.

Sessa, F., Mapelli, M., Ciferri, C., Tarricone, C., Areces, L. B., Schneider, T. R., Stukenberg, P. T., and Musacchio, A. (2005). Mechanism of Aurora B activation by INCENP and inhibition by hesperadin. Mol Cell *18*, 379-391.

Sharif, B., Na, J., Lykke-Hartmann, K., McLaughlin, S. H., Laue, E., Glover, D. M., and Zernicka-Goetz, M. (2010). The chromosome passenger complex is required for fidelity of chromosome transmission and cytokinesis in meiosis of mouse oocytes. J Cell Sci *123*, 4292-4300.

Shigeishi, H., Oue, N., Kuniyasu, H., Wakikawa, A., Yokozaki, H., Ishikawa, T., and Yasui, W. (2001). Expression of Bub1 gene correlates with tumor proliferating activity in human gastric carcinomas. Pathobiology *69*, 24-29.

Shih, I. M., Zhou, W., Goodman, S. N., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (2001). Evidence that genetic instability occurs at an early stage of colorectal tumorigenesis. Cancer Res *61*, 818-822.

Silkworth, W. T., and Cimini, D. (2012). Transient defects of mitotic spindle geometry and chromosome segregation errors. Cell Div *7*, 19.

Skoufias, D. A., Andreassen, P. R., Lacroix, F. B., Wilson, L., and Margolis, R. L. (2001). Mammalian mad2 and bub1/bubR1 recognize distinct spindle-attachment and kinetochore-tension checkpoints. Proc Natl Acad Sci U S A *98*, 4492-4497.

Slattery, S. D., Moore, R. V., Brinkley, B. R., and Hall, R. M. (2008). Aurora-C and Aurora-B share phosphorylation and regulation of CENP-A and Borealin during mitosis. Cell Cycle *7*, 787-795.

Smith, S. L., Bowers, N. L., Betticher, D. C., Gautschi, O., Ratschiller, D., Hoban, P. R., Booton, R., Santibáñez-Koref, M. F., and Heighway, J. (2005). Overexpression of aurora B kinase (AURKB) in primary non-small cell lung carcinoma is frequent, generally driven from one allele, and correlates with the level of genetic instability. Br J Cancer *93*, 719-729.

Sorrentino, R., Libertini, S., Pallante, P. L., Troncone, G., Palombini, L., Bavetsias, V., Spalletti-Cernia, D., Laccetti, P., Linardopoulos, S., Chieffi, P.*, et al.* (2005). Aurora B overexpression associates with the thyroid carcinoma undifferentiated phenotype and is required for thyroid carcinoma cell proliferation. J Clin Endocrinol Metab *90*, 928-935.

Sotillo, R., Hernando, E., Díaz-Rodríguez, E., Teruya-Feldstein, J., Cordón-Cardo, C., Lowe, S. W., and Benezra, R. (2007). Mad2 overexpression promotes aneuploidy and tumorigenesis in mice. Cancer Cell *11*, 9-23.

Steigemann, P., Wurzenberger, C., Schmitz, M. H., Held, M., Guizetti, J., Maar, S., and Gerlich, D. W. (2009). Aurora B-mediated abscission checkpoint protects against tetraploidization. Cell *136*, 473-484.

Storchová, Z., Breneman, A., Cande, J., Dunn, J., Burbank, K., O'Toole, E., and Pellman, D. (2006). Genome-wide genetic analysis of polyploidy in yeast. Nature *443*, 541-547.

Sudakin, V., Chan, G. K., and Yen, T. J. (2001). Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. J Cell Biol *154*, 925-936.

Sugiyama, K., Sugiura, K., Hara, T., Sugimoto, K., Shima, H., Honda, K., Furukawa, K., Yamashita, S., and Urano, T. (2002). Aurora-B associated protein phosphatases as negative regulators of kinase activation. Oncogene *21*, 3103-3111.

Sumara, I., Quadroni, M., Frei, C., Olma, M. H., Sumara, G., Ricci, R., and Peter, M. (2007). A Cul3-based E3 ligase removes Aurora B from mitotic chromosomes, regulating mitotic progression and completion of cytokinesis in human cells. Dev Cell *12*, 887-900.

Sun, L., Gao, J., Dong, X., Liu, M., Li, D., Shi, X., Dong, J. T., Lu, X., Liu, C., and Zhou, J. (2008). EB1 promotes Aurora-B kinase activity through blocking its inactivation by protein phosphatase 2A. Proc Natl Acad Sci U S A *105*, 7153-7158.

Suzuki, A., Ito, T., Kawano, H., Hayashida, M., Hayasaki, Y., Tsutomi, Y., Akahane, K., Nakano, T., Miura, M., and Shiraki, K. (2000). Survivin initiates procaspase 3/p21 complex formation as a result of interaction with Cdk4 to resist Fas-mediated cell death. Oncogene *19*, 1346-1353.

Tanaka, S., Arii, S., Yasen, M., Mogushi, K., Su, N. T., Zhao, C., Imoto, I., Eishi, Y., Inazawa, J., Miki, Y., and Tanaka, H. (2008). Aurora kinase B is a predictive factor for the aggressive recurrence of hepatocellular carcinoma after curative hepatectomy. Br J Surg *95*, 611-619.

Tanaka, T., Kimura, M., Matsunaga, K., Fukada, D., Mori, H., and Okano, Y. (1999). Centrosomal kinase AIK1 is overexpressed in invasive ductal carcinoma of the breast. Cancer Res *59*, 2041-2044.

Tanaka, T. U., Rachidi, N., Janke, C., Pereira, G., Galova, M., Schiebel, E., Stark, M. J., and Nasmyth, K. (2002). Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. Cell *108*, 317-329.

Tang, C. J., Lin, C. Y., and Tang, T. K. (2006). Dynamic localization and functional implications of Aurora-C kinase during male mouse meiosis. Dev Biol *290*, 398-410.

Tang, Z., Bharadwaj, R., Li, B., and Yu, H. (2001). Mad2-Independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1. Dev Cell *1*, 227-237.

Tao, Y., Zhang, P., Girdler, F., Frascogna, V., Castedo, M., Bourhis, J., Kroemer, G., and Deutsch, E. (2008). Enhancement of radiation response in p53-deficient cancer cells by the Aurora-B kinase inhibitor AZD1152. Oncogene *27*, 3244-3255.

Tardáguila, M., González-Gugel, E., and Sánchez-Pacheco, A. (2011). Aurora kinase B activity is modulated by thyroid hormone during transcriptional activation of pituitary genes. Mol Endocrinol *25*, 385-393.

Tatsuka, M., Katayama, H., Ota, T., Tanaka, T., Odashima, S., Suzuki, F., and Terada, Y. (1998). Multinuclearity and increased ploidy caused by overexpression of the aurora- and Ipl1-like midbody-associated protein mitotic kinase in human cancer cells. Cancer Res *58*, 4811-4816.

Taylor, S., and Peters, J. M. (2008). Polo and Aurora kinases: lessons derived from chemical biology. Curr Opin Cell Biol *20*, 77-84.

Terada, Y., Tatsuka, M., Suzuki, F., Yasuda, Y., Fujita, S., and Otsu, M. (1998). AIM-1: a mammalian midbody-associated protein required for cytokinesis. EMBO J *17*, 667-676.

Thompson, S. L., and Compton, D. A. (2010). Proliferation of aneuploid human cells is limited by a p53-dependent mechanism. J Cell Biol *188*, 369-381.

Tomasini, R., Mak, T. W., and Melino, G. (2008). The impact of p53 and p73 on aneuploidy and cancer. Trends Cell Biol *18*, 244-252.

Torres, E. M., Williams, B. R., and Amon, A. (2008). Aneuploidy: cells losing their balance. Genetics *179*, 737-746.

Trakala, M., Fernández-Miranda, G., Pérez de Castro, I., Heeschen, C., and Malumbres, M. (2013). Aurora B prevents delayed DNA replication and premature mitotic exit by repressing p21(Cip1). Cell Cycle *12*, 1030-1041.

Trinkle-Mulcahy, L., Andersen, J., Lam, Y. W., Moorhead, G., Mann, M., and Lamond, A. I. (2006). Repo-Man recruits PP1 gamma to chromatin and is essential for cell viability. J Cell Biol *172*, 679-692.

Turner, B. M. (2002). Cellular memory and the histone code. Cell *111*, 285-291.

Ulisse, S., Delcros, J. G., Baldini, E., Toller, M., Curcio, F., Giacomelli, L., Prigent, C., Ambesi-Impiombato, F. S., D'Armiento, M., and Arlot-Bonnemains, Y. (2006). Expression of Aurora kinases in human thyroid carcinoma cell lines and tissues. Int J Cancer *119*, 275-282.

Vader, G., Medema, R. H., and Lens, S. M. (2006). The chromosomal passenger complex: guiding Aurora-B through mitosis. J Cell Biol *173*, 833-837.

Vischioni, B., Oudejans, J. J., Vos, W., Rodriguez, J. A., and Giaccone, G. (2006). Frequent overexpression of aurora B kinase, a novel drug target, in non-small cell lung carcinoma patients. Mol Cancer Ther *5*, 2905-2913.

Wang, X., Zhou, Y. X., Qiao, W., Tominaga, Y., Ouchi, M., Ouchi, T., and Deng, C. X. (2006). Overexpression of aurora kinase A in mouse mammary epithelium induces genetic instability preceding mammary tumor formation. Oncogene *25*, 7148-7158.

Weaver, B. A., and Cleveland, D. W. (2006). Does aneuploidy cause cancer? Curr Opin Cell Biol *18*, 658-667.

Weaver, B. A., and Cleveland, D. W. (2009). The role of aneuploidy in promoting and suppressing tumors. J Cell Biol *185*, 935-937.

Weaver, B. A., Silk, A. D., Montagna, C., Verdier-Pinard, P., and Cleveland, D. W. (2007). Aneuploidy acts both oncogenically and as a tumor suppressor. Cancer Cell *11*, 25-36.

WEIR, J. B. (1949). New methods for calculating metabolic rate with special reference to protein metabolism. J Physiol *109*, 1-9.

Wiseman, S. M., Masoudi, H., Niblock, P., Turbin, D., Rajput, A., Hay, J., Bugis, S., Filipenko, D., Huntsman, D., and Gilks, B. (2007). Anaplastic thyroid carcinoma: expression profile of targets for therapy offers new insights for disease treatment. Ann Surg Oncol *14*, 719-729.

Wu, C. C., Yang, T. Y., Yu, C. T., Phan, L., Ivan, C., Sood, A. K., Hsu, S. L., and Lee, M. H. (2012). p53 negatively regulates Aurora A via both transcriptional and posttranslational regulation. Cell Cycle *11*, 3433-3442.

Wu, L., Ma, C. A., Zhao, Y., and Jain, A. (2011). Aurora B interacts with NIR-p53, leading to p53 phosphorylation in its DNA-binding domain and subsequent functional suppression. J Biol Chem *286*, 2236-2244.

Yamada, M., Hirotsune, S., and Wynshaw-Boris, A. (2010). The essential role of LIS1, NDEL1 and Aurora-A in polarity formation and microtubule organization during neurogensis. Cell Adh Migr *4*, 180-184.
Yamauchi, T., Uzui, K., Shigemi, H., Negoro, E., Yoshida, A., and Ueda, T. (2013). Aurora B inhibitor barasertib and cytarabine exert a greater-than-additive cytotoxicity in acute myeloid leukemia cells. Cancer Sci *104*, 926-933.

Yan, X., Cao, L., Li, Q., Wu, Y., Zhang, H., Saiyin, H., Liu, X., Zhang, X., Shi, Q., and Yu, L. (2005). Aurora C is directly associated with Survivin and required for cytokinesis. Genes Cells *10*, 617-626.

Yanai, A., Arama, E., Kilfin, G., and Motro, B. (1997). ayk1, a novel mammalian gene related to Drosophila aurora centrosome separation kinase, is specifically expressed during meiosis. Oncogene *14*, 2943-2950.

Yang, K. T., Li, S. K., Chang, C. C., Tang, C. J., Lin, Y. N., Lee, S. C., and Tang, T. K. (2010). Aurora-C Kinase Deficiency Causes Cytokinesis Failure in Meiosis I and Production of Large Polyploid Oocytes in Mouse. Mol Biol Cell.

Ye, D., Garcia-Manero, G., Kantarjian, H. M., Xiao, L., Vadhan-Raj, S., Fernandez, M. H., Nguyen, M. H., Medeiros, L. J., and Bueso-Ramos, C. E. (2009). Analysis of Aurora kinase A expression in CD34(+) blast cells isolated from patients with myelodysplastic syndromes and acute myeloid leukemia. J Hematop *2*, 2-8.

Yuan, K., Li, N., Huo, Y., Yan, F., Yang, Y., Ward, T., Jin, C., and Yao, X. (2009). Recruitment of separase to mitotic chromosomes is regulated by Aurora B. Cell Cycle *8*, 1433-1443.

Zeitlin, S. G., Shelby, R. D., and Sullivan, K. F. (2001). CENP-A is phosphorylated by Aurora B kinase and plays an unexpected role in completion of cytokinesis. J Cell Biol *155*, 1147-1157.

Zekri, A., Lesan, V., Ghaffari, S. H., Tabrizi, M. H., and Modarressi, M. H. (2012). Gene amplification and overexpression of Aurora-C in breast and prostate cancer cell lines. Oncol Res *20*, 241-250.

Zhang, D., Hirota, T., Marumoto, T., Shimizu, M., Kunitoku, N., Sasayama, T., Arima, Y., Feng, L., Suzuki, M., Takeya, M., and Saya, H. (2004). Cre-loxP-controlled periodic Aurora-A overexpression induces mitotic abnormalities and hyperplasia in mammary glands of mouse models. Oncogene *23*, 8720-8730.

Zhang, D., Shimizu, T., Araki, N., Hirota, T., Yoshie, M., Ogawa, K., Nakagata, N., Takeya, M., and Saya, H. (2008a). Aurora A overexpression induces cellular senescence in mammary gland hyperplastic tumors developed in p53-deficient mice. Oncogene *27*, 4305-4314.

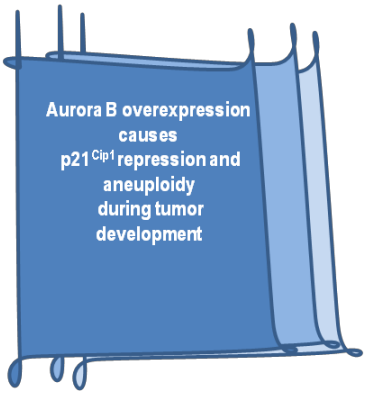
Zhang, H., Chen, X., Jin, Y., Liu, B., and Zhou, L. (2012). Overexpression of Aurora-A promotes laryngeal cancer progression by enhancing invasive ability and chromosomal instability. Eur Arch Otorhinolaryngol *269*, 607-614.

Zhang, N., Ge, G., Meyer, R., Sethi, S., Basu, D., Pradhan, S., Zhao, Y. J., Li, X. N., Cai, W. W., El-Naggar, A. K., *et al.* (2008b). Overexpression of Separase induces aneuploidy and mammary tumorigenesis. Proc Natl Acad Sci U S A *105*, 13033-13038.

Zhang, X., Lan, W., Ems-McClung, S. C., Stukenberg, P. T., and Walczak, C. E. (2007). Aurora B phosphorylates multiple sites on mitotic centromere-associated kinesin to spatially and temporally regulate its function. Mol Biol Cell *18*, 3264-3276.

Zhou, H., Kuang, J., Zhong, L., Kuo, W. L., Gray, J. W., Sahin, A., Brinkley, B. R., and Sen, S. (1998). Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat Genet *20*, 189-193.

Zuazua-Villar, P., Rodriguez, R., Gagou, M. E., Eyers, P. A., and Meuth, M. (2014). DNA replication stress in CHK1-depleted tumour cells triggers premature (S-phase) mitosis through inappropriate activation of Aurora kinase B. Cell Death Dis *5*, e1253.


Aurora B overexpression
causes
p21 Cip1 repression and
aneuploidy
during tumor
development

Appendix

Appendix

Publications derived from this memory:

Fernández-Miranda G, Trakala M, Martín J, Escobar B, González A, Ghyselinck NB, Ortega S, Cañamero M, Pérez de Castro I, Malumbres M. (2011) Genetic disruption of aurora B uncovers an essential role for aurora C during early mammalian development. *Development* 138, 2661-2672.

These two manuscripts contain most of the results presented in this Thesis memory.

González-Loyola A, Fernández-Miranda G,Trakala M,Partida D, Samejima K, Ogawa H, de Cárcer G, Cañamero M, de Martino A,Martínez-Ramírez A, Pérez de Castro I, Earnshaw WC,[2] and Malumbres M. Aurora B overexpression causes $p21^{Cip1}$ repression and aneuploidy during tumor development (*Under review in MCB).*

González-Loyola A, Fernández-Miranda G, Trakala M,Partida D, Cañamero M, de Martino A,Martínez-Ramírez A, Pérez de Castro I, and Malumbres M. Aurora B *in vivo* depletion causes premature ageing due to lack of stem cell regeneration and aneuploidy (*In preparation)*.